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(54) **NOVEL PHOTOACTIVABLE FLUORESCENT DYES FOR OPTICAL MICROSCOPY AND IMAGING TECHNIQUES**

NEUE FOTOAKTIVIERBARE FLUORESZENZFARBSTOFFE FÜR DIE OPTISCHE MIKROSKOPIE UND BILDGEBUNGSVERFAHREN

NOUVEAUX COLORANTS FLUORESCENTS PHOTO-ACTIVABLES POUR DES TECHNIQUES DE MICROSCOPIE OPTIQUE ET D'IMAGERIE

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- **GUNZENHAUSER S., BALLI H.: "Halochromic molecules. Substituted 6,11-dihydrospiro[[1]benzopyrano[4,3-b]indole-6,9'-(9'H)-xanthene]-2',6'- diamines and their aza analogs: new chromogens for black images" HELVETICA CHIMICA ACTA, VERLAG HELVETICA CHIMICA ACTA, BASEL, CH LNKD-DOI:10.1002/HLCA.19900730216, vol. 73, no. 2, 1 January 1990 (1990-01-01), pages 359-379, XP002459316 ISSN: 0018-019X**
- **BELOV V. N., WURM C. A., BOYARSKIY V. P., JAKOBS S., HELL W. H.: "Rhodamines NN: A novel class of caged fluorescent dyes" ANGEWANDTE CHEMIE - INTERNATIONAL EDITION, vol. 49, no. 20, 3 May 2010 (2010-05-03), pages 3520-3523, XP002603279 WILEY-VCH VERLAG DEU DOI: 10.1002/ANIE.201000150**

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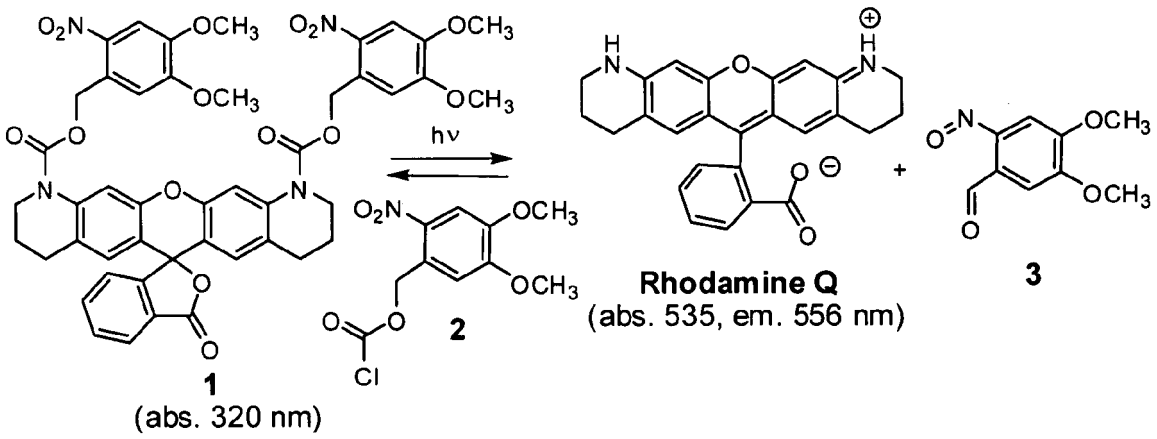
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Description

Background of the invention

[0001] Masked (or "caged") fluorescent dyes initially exist in a non-fluorescent form, which may be transformed into the fluorescent state ("uncaged") by an external stimulus, i. e. enzyme, light, change of the pH-value, etc. This process my be reversible or irreversible, but in most cases "uncaging" means an irreversible change in the course of which the initially colourless or only slightly yellowish substance turns to be strongly coloured and fluorescent. Light is a treasured stimulus in life sciences for several reasons. It may be applied non-invasively, at various wavelengths, with various powers, and with a very high spatial and temporal precision. Therefore, photochemically induced uncaging processes comprise an important tool-box in life sciences and biophysical chemistry. The initially invisible "caged" fluorescent dye may be randomly distributed in the studied object, or it may be used as a label, e. g. in the form of a bioconjugate which "recognizes" the target. After that, a certain dose of UV or visible light produces the coloured and fluorescent species, so that their spatial and temporal distribution may be controlled with high precision at the moment of "uncaging" and later, as the system evolves. Such "optical injections" may generate fluorescent marks not only in biological systems, but also in capillars of the microfluidic sytems [P. H. Paul, M. G. Garguilo, D. J. Rakestraw, Anal. Chem. 1998, 70, 2459-2467; J. I. Molho, A. E. Herr, B. P. Mosier, J. G. Santiago, T. W. Kenny, R. A. Brennen, G. Gordon, B. Mohammadi, Anal. Chem. 2001, 73, 1350-1360]. Imaging of the newly created fluorescent mark followed by its monitoring in time provides quantitative dynamic and structural parameters. For choosing fluorophores for caging, several parameters are important: uncaging efficiency under standard illumination conditions (diode lasers or lamps at wavelengths >375 nm), quick photoactivation (in the range of milliseconds or faster) for tracking rapid processes, high fluorescence quantum yields and photostability after uncaging, ability to penetrate into living cells (alone and after binding with "small" molecules which recognize target structures), low (photo)toxicity of the caged substances and their (decomposition) products after uncaging, high performance in water and aqueous buffers, reliable synthesis and conjugation protocols, stability of bioconjugates against hydrolysis, availability of the multicolor "tool-box" with well separated absorption and emission bands for the (co)localization and FRET studies [*cf.:* R. C. Willis, Anal. Chem. 2007, 79, 1785-1788].

[0002] Coumarines [W.-H. Li, YuRui Zhao, US 7304168 (04.12. 2007); YuRui Zhao, Q. Zheng, K. Dakin, K. Xu, M. Martinez, W.-H. Li, J. Am. Chem. Soc. 2004, 126, 4653-4663], fluorescein [a) R. P. Haugland, K. R. Gee, US 5635608 (03.06.1997); b) T. Kobayashi, Y. Urano, M. Kamiya, T. Ueno, H. Kojima, T. Nagano, J. Am. Chem. Soc. 2007, 129, 6696-6697; c) G. A. Krafft, W. R. Sutton, R. T. Cummings, J. Am. Chem. Soc. 1988, 110, 301-303] and rhodamines [a) Rhodamine Q: T. J. Mitchison, K. E. Sawin, J. A. Theriot, K. Gee, A. Mallavarapu, Methods in Enzymology 1998, 291, 63-78; Bioorg. Med. Chem. Lett. 2001, 11, 2181-2183; b) Rhodamine 110: L. D. Lavis, T.-Y. Chao, R. T. Raines, ACS Chem. Biol. 2006, 1, 252; J. Ottl, D. Gabriel, G. Marriott, Bioconjugate Chem. 1998, 9, 143-151] have been disclosed and used as caged fluorescent dyes. Synthesis and properties of caged fluorescent 2-amidothioxanthones - compounds structurally similar to fluorescein or rhodamines - were also reported [J. R. R. Majjigapu, A. N. Kurchan, R. Kattani, T. P. Gustafson, A. G. Kutateladze, J. Am. Chem. Soc. 2005, 127, 12458-12459]. As a photocleavable unit, most of these caged compounds contain a 2-nitrobenzyl group or its derivatives with an α-substituent and/or one or two methoxy groups in the aromatic ring [US 5635608]. The α-position to the phenyl ring (CH_2-group) may be decorated with a carboxy or an alkyl group facilitating the uncaging reaction. An example of the caged Rhodamine Q is given below. This model compound (compound **1**) was synthesized by the present inventors as indicated in Scheme 1.



Scheme 1. Photolysis of the caged rhodamine Q (compound **1**) with a mercury arc lamp (>360 nm) or with a diode laser (375 nm) produces the fluorescent dye and 4,5-dimethoxy-2-nitrosobenzaldehyde (**2**).

[0003] Methoxy groups in combination with a nitro group in the aromatic ring provide the required absorption in the near UV region and also speed-up the liberation of an uncaged fluorescent dye. Without them the removal of 2-nitrobenzyl group is very slow and requires hard UV light incompatible with living cells. Acylation of both nitrogen atoms in rhodamine Q with commercially available compound **2** affords the yellow compound **1** with a spiro junction of the xanthene fragment and the aromatic lactone ring. In this non-fluorescent compound the extended π-conjugation is broken, and therefore the deep red colour of rhodamine Q disappeared. Practical applications (e. g. bioconjugation) require compounds with a free second carboxy group or other functionality in the disubstituted "lower" benzene ring. However, the synthesis of all caged rhodamines with such a second carboxy group is difficult and low-yielding [T. J. Mitchison, K. E. Sawin, J. A. Theriot, K. Gee, A. Mallavarapu, Methods in Enzymology 1998, 291, 63-78]. Probably because of that they did not find any widespread use.

[0004] A big disadvantage of 2-nitrobenzyl group and its substitutes is that upon photolysis they produce colored and highly reactive 2-nitrosobenzaldehyde or 2-nitrosobenzophenone. All aromatic nitroso compounds including 2-nitrosobenzaldehyde, 2-nitrosobenzophenone and their derivatives are known to be toxic. Acute toxicity of these compounds may unfavourably influence the normal cell life, and even cause cell death. The chemical reactivity of 2-nitrosobenzaldehyde or 2-nitrosobenzophenone is very high; they react with amino groups in proteins giving Schiff's bases (imines) which form oligomers, especially under irradiation with UV-light. The dark colour of these oligomeric by-products may interfere with optical measurements; they may act as a sort of undesired optical filter. The amount of these by-products and oligomers could be reduced, if it were possible to use the monoacylated rhodamine derivatives. Unfortunately, they are still fluorescent (though they absorb and emit at shorter wavelengths than the free dye). An urgent need for other caging groups free from the drawbacks mentioned above stimulates the on-going research. However, other modern caging groups which absorb in the near UV region are also bulky, and the procedures of their synthesis, caging and uncaging reactions may be rather complicated. For example, *N,N*-dimethyl-2-hydroxy-4-nitrophenol was reported to give photocleavable phenyl esters [A. Banerjee, C. Grewer, L. Ramakrishnan, J. Jäger, A. Gameiro, H.-G. A. Breitinger, K. R. Gee, B. K. Carpenter, G. P. Hess, J. Org. Chem. 2003, 68, 8361-8367; *cf.:* M. Matsuzaki, G. C. R. Ellis-Davies, T. Nemoto, Y. Miyashita, M. Iino, H. Kasai, Nature Neurosci. 2001, 4, 1086-1092]. Another heterocycle - 7-diethylamino-4-(hydroxymethyl)-2*H*-chromen-2-one - is known to form esters which may easily be cleaved by irradiation at 412 nm [P. Stegmaier, J. M. Alonso, A. Del Campo, Langmuir 2008, 24, 11872-11879]. Yet other heterocycles - derivatives of 8-bromo-7-hydroxyquinoline [O. D. Fedoryak, T. M. Dore, Org. Lett. 2002, 4, 3419-3422] and 6-bromo-7-hydroxycoumarines [T. Furuta, S. S.-H. Wang, J. L. Dantzker, T. M. Dore, W. J. Bybee, E. M. Callaway, W. Denk, R. Y. Tsien, Proc. Natl. Acad. Sci. USA 1999, 96, 1193-2000; H. Ando, T. Furuta, R. Y. Tsien, H. Okamoto, Nat. Genet. 2001, 28, 317-325; W. Lin, D. S. Lawrence, J. Org. Chem. 2002, 67, 2723-2726; H. J. Montgomery, B. Perdicakis, D. Fishlock, G. A. Lajoie, E. Jervis, J. G. Guilemette, Bioorg. Med. Chem. 2002, 10, 1919-1927] - have also been proposed as photocleavable protecting groups. All of these groups provide the required absorption in the near UV or violet spectral region and may be cleaved off by photoirradiation. The general disadvantage of all bulky caging groups is that their big size and limited

solubility in water considerably reduce the cell permeability of the whole assemblies with fluorescent dyes. Further conjugation with small molecules is sometimes required for selective binding with the biological targets. These additional structural fragments further increase the molecular dimensions and may retard or even inhibit the penetration of the whole photoactivable adducts through cell walls and bio-membranes precluding their successful use in biological microscopy.

[0005] Gunzenhauser et al., in Helvetica Chimica Acta, Vol. 62, 171-184 (1979), describe heterospiran-xanthene derivatives which, however, do not contain any chromophore moieties capable to eliminate N_2 or N_2O molecules after photolysis by irradiation. The same authors disclose, in Helvetica Chimica Acta, Vol. 73, 359-379 (1990), xanthene benzopyrano derivatives which compounds are structurally quite different from rhodamines and carbopyronines.

[0006] In WO 92/08720 A1 photolabile rhodamine derivatives suitable for labelling purposes are disclosed which after photolysis release 2-nitrobenzyl groups.

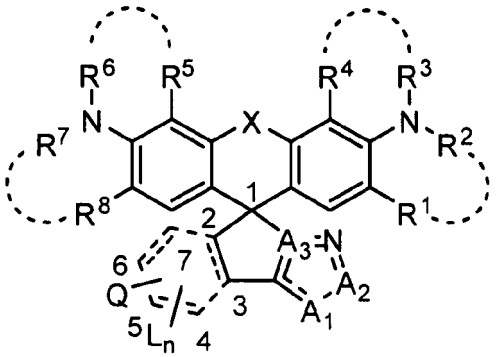
[0007] In view of this prior art , the main object of the present invention was to provide novel photoactivable fluorescent dyes incorporating small photoactive groups with improved properties such as the capability for easy and effective photoactivation (uncaging) which does not generate toxic substances which may interfere with biochemical processes in cells or tissues.

[0008] A related object was to provide novel reagents and methods for biocojugation and various imaging techniques, including those which provide optical single molecule switching (SMS) "nanoscopy" (diffraction unlimited optical resolution by using switching of the fluorescence of the single molecules).

[0009] These objectives have been achieved by providing the novel photoactivable compounds according to claims 1-3, the methods of preparation according to claims 4-5 and the compounds for the specific uses of claims 6-19.

Description of the Invention

[0010] The novel photoactivable compounds according to claim 1 are photoactivable rhodamine or carbopyronine derivatives of the following general formulae **G1-G4** comprising UV light absorbing chromophores which after photolysis by irradiation at 254-490 nm (preferably at 375-420 nm) generate fluorescent rhodamine or carbopyronine derivatives and small non-toxic fragments such as N_2 or N_2O.

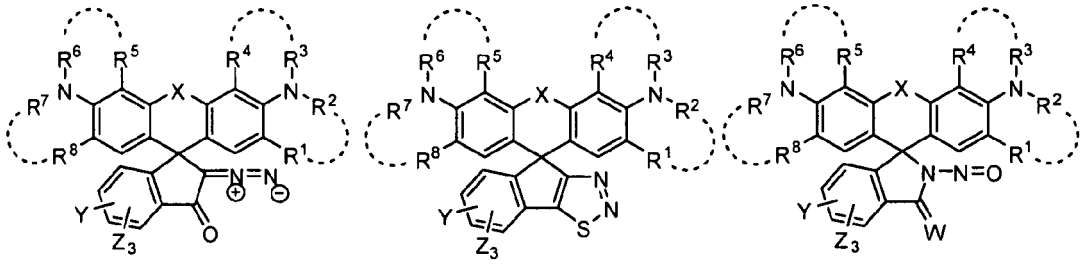


G1 - G4

G1: A1 = 0, A2 = N, A3 = C;
G2: A1 = S, A2 = N, A3 = C;
G3: A1 = O, A2 = O, A3 = N;
G4: A1 = S, A2 = O, A3 = N;
wherein R^1, R^2, (R^1-R^2), R^3, R^4, (R^3-R^4) , R^5, R^6, (R^5-R^6) , R^7, R^8, (R^7-R^8) = H, unsubstituted or substituted alkyl (cycloalkyl), unsubstituted or substituted cycloalkenyl, unsubstituted or substituted heterocylic residue, unsubstituted or substituted aryl, unsubstituted or substituted hetaryl; and X = O, S, C $(CH_3)_2$;
the chemical bond between C-2 and C-3 may be a single or double bond (more specifically a *cis*-C(Q)=C(L)-bond); alternatively, C-2 and C-3 may be a part of any cyclic system, preferably the benzene ring;
and the substituients Q and/or L are independently defined as follows (*n* = 0, 1, 2, 3 or 4) : H, $COOR^9$, COCH=N=N, CH_2COOR^9, N (R^1···R^2), $NR^{10}R^{11}$, SR^{12}, F, Cl ;
wherein R^9 = H, unsubstituted or substituted alkyl, cycloalkyl, or heterocyclic residue, *N*-succinimidyl, *N*-sulfosuccinimidyl, unsubstituted or substituted phenyl, aryl or hetaryl; R^{10}, R^{11} = C=S, C=O ; R^{12} = H, $(CH_2)_mCOOR^9$ (*m* = 1 - 11), unsubstituted or substituted alkyl, cycloalkyl, heterocyclic residue, aryl or hetaryl.

[0011] In a preferred embodiment of the present invention, the novel photoactivable compounds of the invention are

represented by the general structural formulae **I, II and III** below



Scheme 2. Photoactivable 9*H*-xanthene (X = O) or 9,10-dihydroanthracene (X = CMe_2) derivatives with spiro 2-diazoketone (**I**), 1,2,3-thiadiazole (**II**), and *N*-nitroso(thio)amide (**III**) fragments; wherein R^1, R^2, (R^1–$R^{2)}$, R^3, R^4, (R^3–R^4), R^5, R^6, (R^5–R^6), R^7, R^8, (R^7–R^8) = H, unsubstituted or substituted alkyl (cycloalkyl), unsubstituted or substituted cycloalkenyl, unsubstituted or substituted heterocylic residue, unsubstituted or substituted aryl, unsubstituted or substituted hetaryl; and X = O, S, $C(CH_3)_2$; Y = $COOR^9$, COCH=N=N, CH_2COOR^9, $N(R^1 \cdots R^2)$, $NR^{10}R^{11}$, SR^{12}, F, Cl; Z = H, F, Cl; W = O, S; R^9 = H, unsubstituted or substituted alkyl, cycloalkyl, or heterocyclic residue, *N*-succinimidyl, *N*-sulfosuccinimidyl, unsubstituted or substituted phenyl, aryl or hetaryl; R^{10},R^{11} = C=S, C=O; R^{12} = H, $(CH_2)_mCOOR^9$ (*m* = 1 - 11), unsubstituted or substituted alkyl, cycloalkyl, heterocyclic residue, aryl or hetaryl.

[0012] The term "heterocyclic residue", as used herein, refers to an unsubstituted or substituted mono-, bi-, tri- or polycyclic ring system incorporating the groups R^1-R^2 and/or R^3-R^4 and/or R^5-R^6 and/or R^7-R^8 fused with the 3, 6-diamino spiro-9*H*-xanthene or 9,10-dihydroanthracene fragment in such a way that these groups R^1 and R^2, (and/or R^3 and R^4, R^5 and R^6, R^7 and R^8) are bound with each other directly or through any additional bridge (linker), which may contain or may not contain any additional heteroatom (e. g. Si, N, O, P, S, Se, etc.).

[0013] The term "aryl", as used herein, refers to an unsubstituted or substituted mono-, bi- or tricyclic carbocyclic ring system having one, two or three aromatic rings including but not limited to phenyl, naphthyl, anthryl, azulyl, tetrahydronaphthyl, indanyl and indenyl.

[0014] The term "hetaryl", as used herein, refers to an unsubstituted or substituted cyclic aromatic radical having from 5 to 10 ring atoms of which at least one ring atom is selected from S, O and N; the radical being joined to the rest of the molecule via any of the ring atoms. Representative, but not limiting examples are pyridinyl, pyrazinyl, pyrimidinyl, pyrrolyl, imidazolyl, thiazolyl, oxazolyl, isooxazolyl, thiadiazolyl, oxadiazolyl, thiophenyl, furanyl, quinolinyl and isoquinolinyl.

[0015] The terms "alkyl" or "cycloalkyl", as used herein, comprise any unsubstituted or substituted (cyclo)alkyl groups. Specific, but not limiting examples are an unsubstituted or substituted methyl group, ethyl group, lower (cyclo)alkyl group with 3-10 C atoms, or (cyclo)alkyl group with 11-30 or more C atoms.

[0016] The term "cycloalkenyl", as used herein, comprises any unsubstituted or substituted cycloalkenyl groups. Spe-

cific, but not limiting examples are unsubstituted or substituted lower cycloalkenyl groups with 3-10 C atoms, or cycloalkenyl groups with 11-30 or more C atoms.

[0017] All compounds of formulae **I-III** comprise the basic rhodamine or carbopyronine scaffolds and have smaller functional groups (chromophores) absorbing UV light.

[0018] Compounds with general formula **I** contain a 2-diazoketone (α-diazoketone) fragment, compounds **II** possess the 1,2,3-thiadiazole heterocycle, and compounds **III** an *N*-nitroso(thio)amide moiety. These residues all consist of only 5 atoms and are photochemically active. Compounds **I-III** are yellow solids and their solutions are non fluorescent. Photolysis at at 254-490 nm (preferably at 375-420 nm) generates highly coloured and fluorescent products - derivatives of rhodamine (X = O) or carbopyronine (X = CMe_2) dyes.

[0019] A further aspect of the invention relates to various advantageous uses of the claimed compounds, in particular of the structural formulae **I-III.** Due to their favourable characteristics, these compounds represent efficient fluorescent dyes and markers.

[0020] The claimed compounds can easily be photoactivated (uncaged) with UV-light (preferably at 375-420 nm). Photodecomposition (uncaging) generates the coloured and highly fluorescent derivatives of rhodamine or carbopyronine fluorescent dyes. The new photoactivable compounds penetrate into the living cells. Irradiation of these compounds is accompanied with elimination of small stable molecules - nitrogen and nitrogen(I) oxide. The uncaging reaction does not generate any toxic substances which may interfere with biochemical processes in cells or tissues. The new caged fluorescent dyes may be coupled with antibodies and used in immunolabelling experiments followed by photoactivation in aqueous (cell culture) media. Uncaging protocols may be implemented with common fluorescent microscopes, optics with high numerical apertures and light sources (diode lasers and lamps). Gradual photoactivation using light with low intensities is also possible. Rapid switching within 1-3 ms allows the tracking of fast processes in cells, thin capillaries, etc. The new masked fluorescent dyes may be used as markers in imaging techniques and other applications where spatial and temporal resolution is essential (e.g. for protein tracking, in microfluidic devices, labs on the chip, etc.). The photoactivable fluorescent dyes of the present invention may be also used in the newly emerged nanoscopic techniques, in which uncaging of single molecules followed by acquisition of the emitted photons and application of various localization protocols allows an image restoration with an optical resolution of ca. 50 nm.

[0021] The novel photactivable compounds can be readily coupled to other molecules, in particular biomolecules such as peptides, proteins, lipids, carbohydrates, nucleic acids, or toxins, and the resulting bioconjugates can be used as fluorescent dyes or markers as well.

[0022] Thus, the present invention provides the use of compounds according to any one of claims 1-3 as reagents for conjugation or bioconjugation. The conjugation or bioconjugation (conjugation with a biomolecule, e.g. a type of molecule which is present in a (living) cell or an organism, including a microorganism or virus) comprises formation of at least one covalent chemical bond or at least one molecular complex with any chemical entity, substance or object, e.g., amine, carboxylic acid, aldehyde, alcohol, aromatic compound, heterocycle (e.g. biotin), dye, amino acid, amino acid residue coupled to any chemical entity, peptide, protein (e. g. avidin, streptavidin), carbohydrate, nucleic acid, toxin, lipid, enzyme, antibody, etc.

[0023] In a more specific embodiment the conjugation comprises the formation of two or more covalent chemical bonds and/or molecular complexes with any chemical entities, substances or objects, e.g., amines, carboxylic acids, aldehydes, alcohols, aromatic compounds, heterocycles (biotin), dyes, amino acids, peptides, proteins (e.g. avidin, streptavidin), carbohydrates, nucleic acids, toxins, lipids, enzymes, antibodies, etc. and the reagents may be cross-linking agents.

[0024] The use of said compounds for conjugation or cross-linking may be in solution, as colloids or solids.

[0025] In a preferred specific embodiment, compounds **22**-NHS, **23**-NHS, **17** and **30** with two functional groups are used as (cross)linking reagents.

[0026] A specific use of the compounds according to claims 1-3 or of their conjugates comprises the use for bioconjugation with (secondary) antibodies and immunolabelling/immunostaining.

[0027] The present invention also provides the use of the compounds of formulae **G1-G4** or **I-III** as such or after photoactivation or of their conjugates with biomolecules or any other chemical entity, substance or object, e.g., amine, carboxylic acid, aldehyde, alcohol, aromatic compound, heterocycle (biotin), dye, amino acid, amino acid residue, peptide, a protein such as e.g. avidin, streptavidin, an enzyme, antibody, a carbohydrate, toxin, lipid, a nucleic acid such as DNA, RNA or a fragment or derivative thereof including an (oligo)nucleotide or (oligo)nucleoside, etc., as (fluorescent) dyes or labels.

[0028] In this use, the compounds in claims 1-3 or their conjugates may be transformed from the caged (masked) non-fluorescent structure to the fluorescent form (uncaged) by UV or visible light which causes a certain chemical reaction to occur, namely the elimination of molecular nitrogen (N_2) or nitrogen(I) oxide (N_2O). Photoactivation (uncaging) of the claimed compounds is performed in solution (water, aqueous buffers, organic solvents and their mixtures), in a solid state, in colloids and polymer matrices. Complete photoactivation within 1 ms - 1 s is feasible. Gradual or step-wise photoactivation is possible with low light intensities. Preferably, starting (caged) compounds are colorless or yellow and possess the long-wave absorption band with a maximum at 250-450 nm and may be uncaged (activated) with diode

lasers irradiating at 375 nm or lamps at wavelengths 250-500 nm.

[0029] Specifically, the present invention provides the use of the compounds of formulae **G1-G4** or **I-III** as such or after photoactivation or of their conjugates as dyes and/or labels in the fields of optical microscopy and imaging techniques, microfluidic devices, capillary electrophoresis, protein tracking techniques. More specifically, the optical microscopy and imaging techniques comprise stimulated emission depletion microscopy [STED], single molecule switching (SMS) "nanoscopy" (diffraction unlimited optical resolution by using switching of the fluorescence of the single molecules: e. g. single molecule localization microscopy [SMLM], photoactivation localization microscopy [PALM, PALMIRA, fPALM], stochastic optical reconstruction microscopy [STORM]), fluorescence correlation spectroscopy [FCS], fluorescence recovery after photobleaching [FRAP], fluorescence lifetime imaging [FLIM], ground state depletion with individual molecular return [GSDIM], and fluorescence resonant energy transfer [FRET], but are not limited thereto.

[0030] Further, the present invention also provides the use of the compounds according to any one of claims 1-3 or of their conjugates as cell permeable substances penetrating through membranes of living and fixed cells in the caged non-fluorescent form which may be later photoactivated (uncaged) inside a living or fixed cell.

[0031] In a still further embodiment, the present invention provides the use of the compounds according to any one of claims 1-3 or of their conjugates as such or after photoactivation for tracking and monitoring dynamic processes in a sample or in an object. Typically, in this embodiment changes in the shape, dimensions and/or the intensity of the fluorescence signal obtained after photoactivation of the compounds according to any one of claims 1-3 or of their conjugates will correspond to a change or evolution of the sample or object in study or of its environment, e.g. protein tracking, flow of a liquid, etc.

[0032] A further specific embodiment relates to the use of the compounds according to any one of claims 1-3 or of their conjugates as such or after photoactivation as labels specific for mitochondria. In particular, compounds **22**-H, **22**-NHS, **23**-H, and **23**-NHS may be advantageously used as labels specific for mitochondria.

[0033] A still further specific embodiment relates to the use of the claimed compounds, in particular of compounds **22**-H, **22**-NHS, **23**-H and **23**-NH in single molecule switching (SMS) nanoscopy. The use of such compounds drastically improves the optical resolution and provides diffraction-unlimited imaging (e.g. with an optical resolution better than 100 nm).

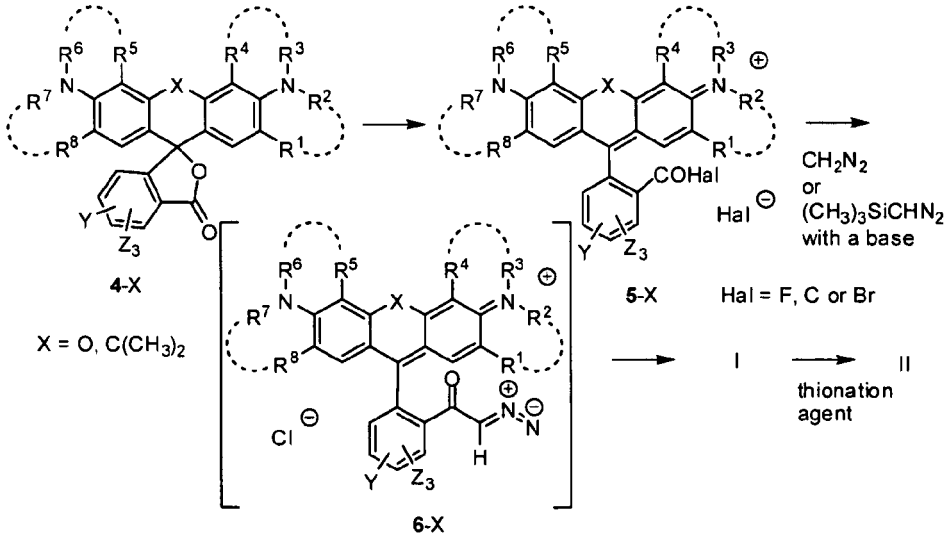
[0034] In a further aspect, the present invention also provides methods for producing compounds of the general formulae **I-III.**

[0035] In a specific embodiment, the following method consisting of steps a), b) and c) for preparing compounds **I** and **II** according to claim 2, is provided:

a) converting the spiro-9*H*-xanthene or 9,10-dihydro-anthracene starting compound **4-X** as depicted in the following scheme (or their synthetically equivalent open forms having a free carboxylic group) into the corresponding acid halide **5-X;**

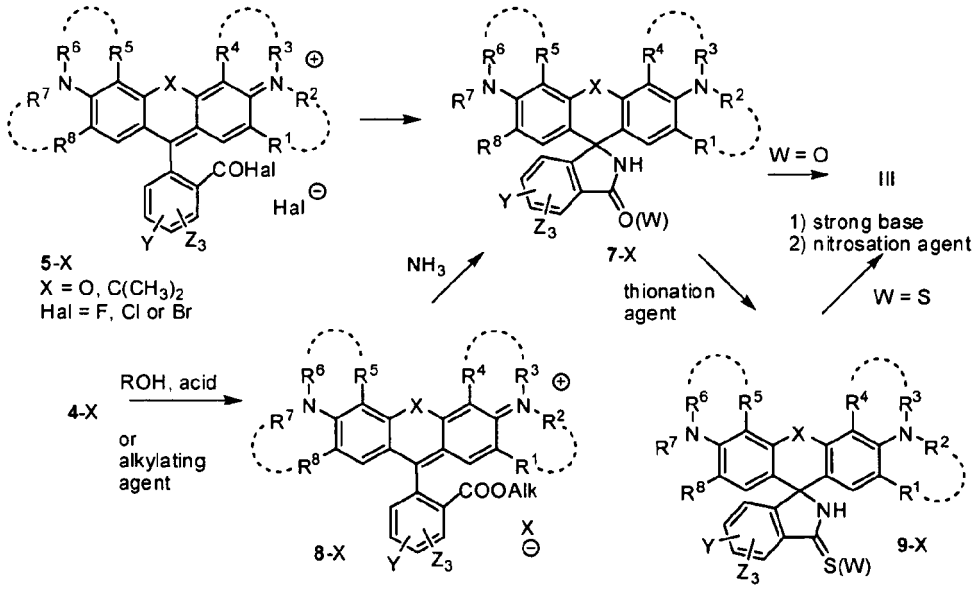
b) reacting compound **5-X** with a diazomethane compound to produce the diazoketone **I;**

c) optionally reacting compound **I** with a thionating agent to produce the 1,2,3-thiadiazole derivative **II.**



[0036] As a further specific embodiment, the following method for preparing compounds **III** according to claim 2, comprising the steps a), b) and c), is disclosed:

a) converting the acid halide **5-X** or alkyl ester **8-X** as depicted in the following scheme into the amide **7-X;**
b) reacting compound **7-X** with a thionation agent to produce the corresponding thioamide **9-X;**
c) deprotonation of the amide **7-X** or thioamide **9-X** with a strong base followed by the reaction with a nitrosation agent.



[0037] In a more specific embodiment, there is provided a method for preparing compounds **I** and **II** according to claim 2, comprising the steps:

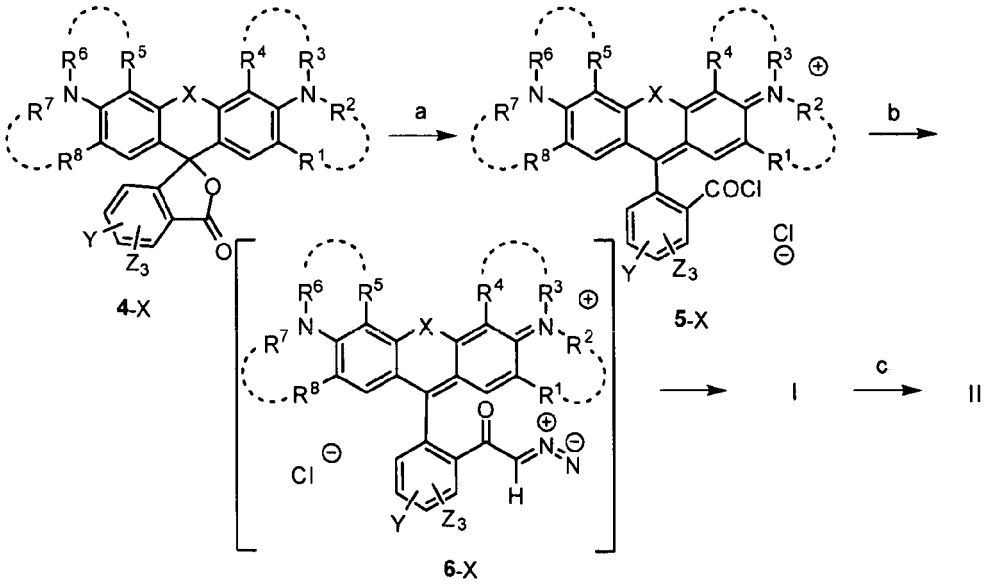
a) converting the spiro-9*H*-xanthene or 9,10-dihydro-anthracene starting compound **4-X** of scheme 3 (or their synthetically equivalent open forms having a free carboxylic group) into the corresponding acid chloride **5-X;**
b) reacting compound **5-X** with a diazomethane compound (represented by diazomethane or trimethylsilyldiazomethane) to produce the diazoketone **I;**
c) optionally reacting compound **I** with a thionating agent (e. g. Lawesson reagent or P_4S_{10}) to produce the 1,2,3-thiadiazole derivative **II.**

[0038] In another more specific embodiment, the present invention provides a method for preparing compounds **III** according to claim 2, comprising the steps:

a) converting the acid chloride **5-X** or the methyl ester **8-X** of scheme 4 into the amide **7-X;**
b) reacting compound **7-X** with a thionation agent (e. g. Lawesson reagent or P_4S_{10}) to produce the corresponding thioamide **9-X;**
c) deprotonation of the amide **7-X** or thioamide **9-X** with a very strong base (e. g. tBuOK, $NaN(SiMe_3)_2$, NaH, KH, super strong (phosphasene) organic bases, etc.) followed by the reaction with a nitrosation agent (e. g. NOCl or NO (+) $*BF_4$ (-)).

General synthesis of the novel photoactivable compounds of the invention

[0039] Synthetic routes to the new photoactivable compounds are given in Schemes 3-10. Rhodamines and carbopyronines are convenient starting materials for the synthesis of the spiro-compounds **I-III** (Schemes 3, 4).



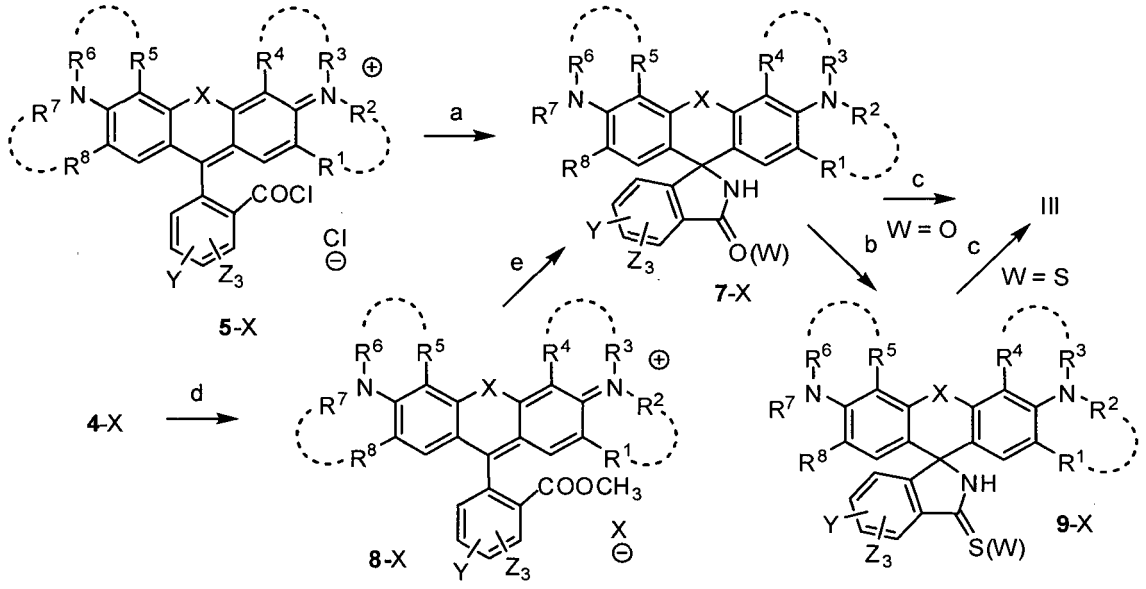
Scheme 3. General approach to spiro 2-diazoketones (**I**) and 1,2,3-thiadiazoles (**II**): a) $(COCl)_2$, CH_2Cl_2, room temp., 2 – 4 h or $POCl_3$ in 1,2-dichloroethane, reflux, 4 h; b) CH_2N_2 or 2 M Me_3SiCHN_2 in Et_2O with Et_3N, THF; c) Lawesson reagent, benzene (toluene) or THF, heating.

[0040] Activation of the free carboxylate in compound **4**-X (Scheme 3) with oxalyl chloride or, for the less reactive substrates, by heating with $POCl_3$ produces the corresponding acid chloride **5**-X. Careful removal of an excess of the reagent followed by the reaction of the acid chloride **5**-X with a solution of diazomethane or commercially available trimethylsilyl diazomethane in ether, directly affords the yellowish and non-fluorescent closed form of an α-diazoketone **I.** Obviously the proton in the CH=N=N group is acidic enough, and, after abstraction of HCl, the open form of diazoketone **6**-X cyclises into the more stable closed isomer **I.** Analogous reaction of the primary rhodamine amides is known and affords the colourless ring-closed isomers.

[0041] α-Benzoyl diazoalkanes have an absorption band with a maximum at about 320 nm and ε ~ 12000. Photolysis of α-diazoketones is accompanied by elimination of nitrogen, and the intermediately formed carbene participates in Wolff rearrangement. It forms the corresponding ketene which is then trapped by any nucleophile available in the reaction medium (water, amine, alcohol, etc.). These transformations result in the elongation of the carbon chain and have been studied in detail [W. Kirmse, Eur. J. Org. Chem. 2002, 2193-2256]. The relevant examples will be given below. Interestingly, photolysis of diazoketone has been used in bioconjugation for cross-linking of the amine containing compounds [G. T. Hermanson *Bioconjugate techniques* (Academic Press, Elsevier) **1996,** 272-274].

[0042] α-Diazo carbonyl compounds may be used as starting materials for other photoreactive substances - 1,2,3-thiadiazole derivatives **II** [M. I. Levinson, M. P. Cava, Heterocycles, 1982, 19, 241-243]. The latter easily undergo photodegradation forming highly reactive thioketenes [P. Krauss, K.-P. Zeller, H. Meier, E. Muller, Tetrahedron 1971, 27, 5953-5957], which also add nucleophiles.

[0043] Another novel small photoactivable group incorporated into the rhodamine or carbopyronine fluorophore is the N-nitrosothioamide group (Scheme 4). Thioamides are known to absorb weakly in the near UV-region [Photochemistry of thioamides and thioimides have been reviewed: a) M. Sakamoto, T. Nishio in: CRC handbook of Organic Photochemistry and Photobiology (2nd Ed.) (Ed.: W. Horspool, F. Lenci), CRC Press LLC, Boca Raton, 2004, pp. 106/1-106/20; b) T. Nishio, M. Sakamoto, Rev. Heteroatom Chem. 1995, 12, 23-52]. Thioamides of rhodamines (without N-nitroso group) are known. ' Primary rhodamine thioamides are known exist in the closed form. For the synthesis of the compound class **III,** it is necessary to prepare the simplest thioamides **9**-X as starting materials (Scheme 4).



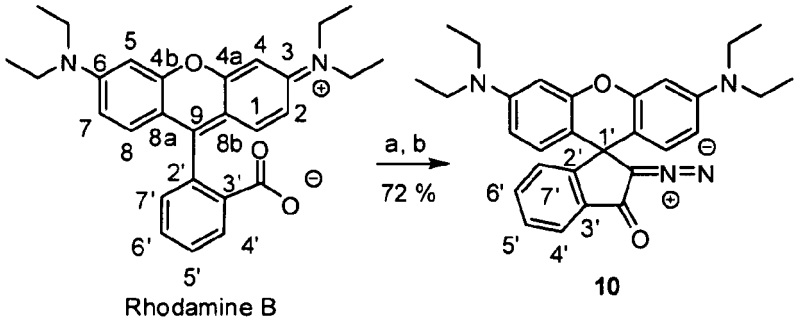
Scheme 4. General approach to spiro N-nitroso(thio)amides (**III**): a) aq. or alcoholic NH_3, MeCN, room temp.; b) Lawesson reagent, benzene (toluene), heating; c) strong base, then NOCl or NO(+)*BF_4(-); d) MeOH, mineral acid, heating; e) aq. or alcoholic NH_3, heating.

[0044] The latter are easily available via two routes which involve either the corresponding acid chlorides **5**-X, or methyl esters **8**-X. Acid chlorides **5**-X are very reactive, and the simplest amides **7**-X may be obtained from them under very mild conditions. Methyl esters **8**-X can also be transformed into the required amides **7**-X, but this transformation is feasible only under the harsh conditions: heating with concentrated alcoholic or aqueous ammonia in an autoclave is normally required to complete the amidation reaction. Amides **7**-X may easily be transformed into the corresponding thioamides **9**-X using the Lawesson reagent (or P_2S_5 in pyridine). After deprotonation with commercially available very strong bases (e. g. *t*BuOK, $NaN(SiMe_3)_2$, NaH, KH, phosphasene organic bases, etc.), amides **7**-X or thioamides **9**-X may undergo *N*-nitrosation with e.g. nitrosyl chloride (NOCl) or NO(+)*BF_4(-). Photodecomposition of *N*-nitroso(thio)amides **III** is accompanied by elimination of nitrogen(I) oxide (N_2O), and the intermediately formed highly reactive thioketenes may add nucleophiles and rearrange to colored and fluorescent products.

[0045] Important limitations associated with these synthetic routes follow from the use of the acid chlorides **5**-X or the nitrosation agents mentioned above. Acid chlorides may acylate the primary and secondary amino groups in rhodamines and carbopyronines. Similarly, nitrosyl chloride or NO(+)*BF_4(-) in the presence of a base may react with these primary and secondary amino groups, too. The primary amino groups and NOCl give the corresponding (unstable) diazonium salts, and the secondary amino groups under these conditions produce N-nitroso amines. Therefore, both amino groups in the starting rhodamines **4**-0 or carbopyronines 4-CMe_2 should be fully substituted. Moreover, to avoid C-nitrosation into the ortho-position to the *N,N*-dialkylamino groups, (thio)amides **7**-X (**9**-X) should be deprotonated (at the nitrogen atom) prior to the exposure to the nitrosation agent. For the same reason, nitrosation with HNO_2 under acidic conditions should be avoided, as it is expected to give C-nitroso derivatives.

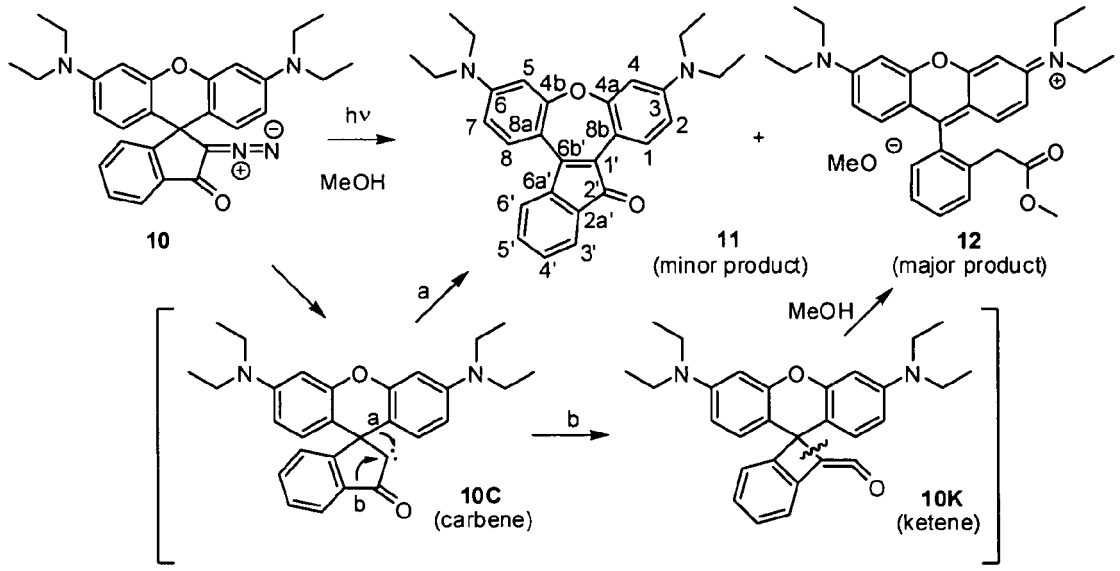
[0046] An important feature of the present invention is that it is based on the new class of 2-diazoketones or their analogues incorporated into the skeleton of fluorescent dyes. The new transformation which was difficult to predict and which provided new useful properties is the spontaneous ring-closing reaction of the intermediates **6**-X into compounds **I.** The cell permeability of the compact spiro compounds **I - III** before photoactivation is expected to be better than that of the corresponding uncaged fluorescent dyes. The reason for this is that the initial substance is neutral and bears no electrical charges, but after elimination of N_2 or N_2O the zwitterionic form of the fluorescent dye forms, which is known to cross the membrane much more reluctantly than the corresponding "closed" (lactone) isomer of the same dye.

[0047] Rhodamine B is an inexpensive model compound for developing the synthetic routes to compound classes **I-III.** The feasibility of the synthesis of benzoyl diazoalkanes **I** is illustrated by Scheme 5. *N,N,N',N'*-Tetramethylrhodamine (TMR) reacts quite similarly and affords the corresponding diazoketone in high yield.



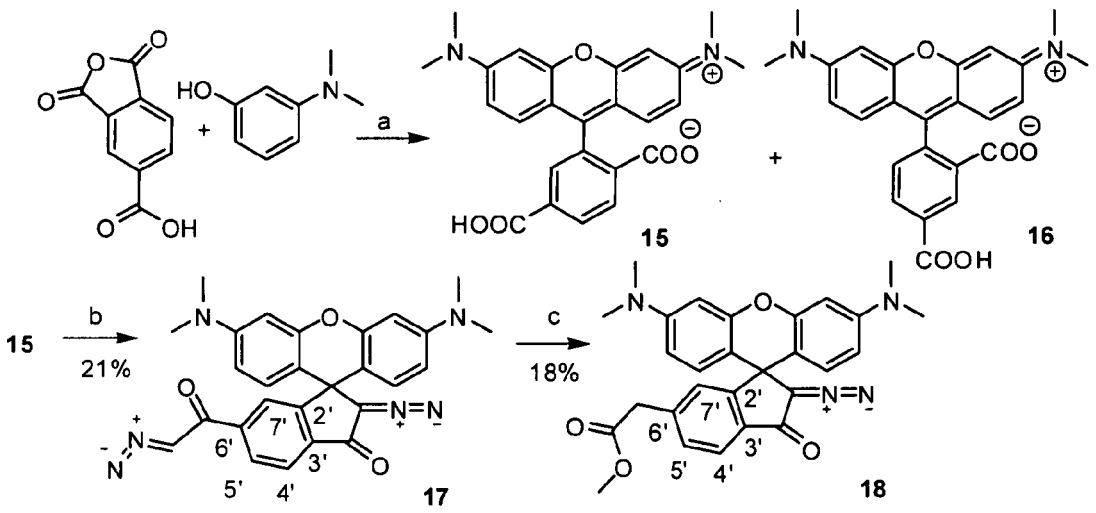
Scheme 5. Conversion of Rhodamine B into the spiro diazoketone **10**: a) $POCl_3$ in 1,2-dichloroethane, reflux, 4 h; b) CH_2N_2 in THF, 0 °C – room temp., 12 h.

[0048] Diazoketone **10** was subjected to photolysis in methanol in order to study the product distribution. Light with wavelengths >320 nm was used. The result of this experiment is presented in Scheme 6.



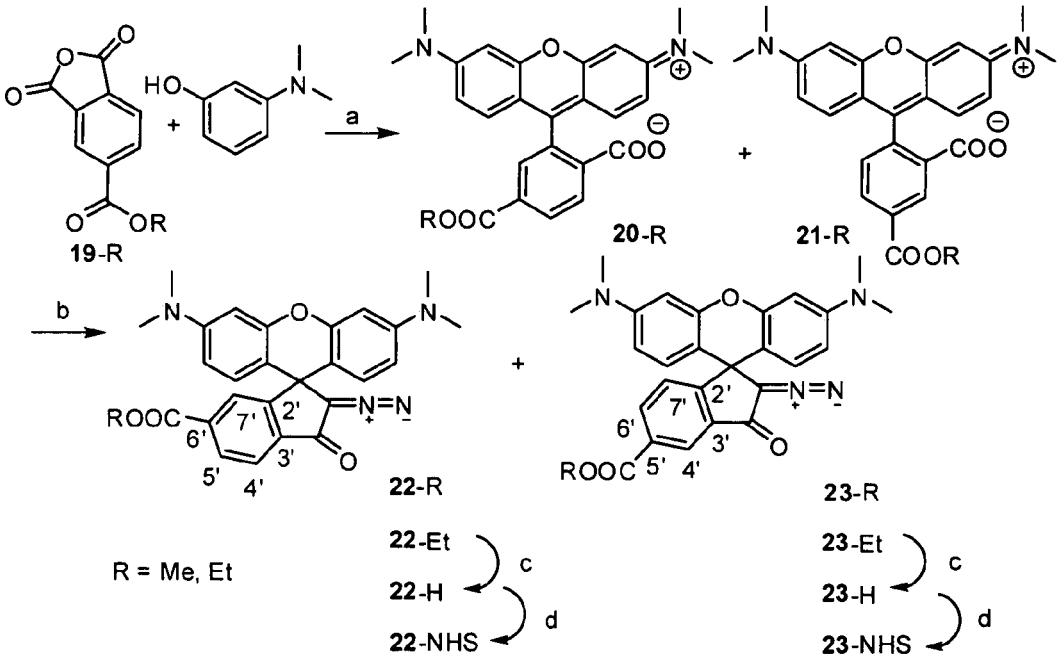
Scheme 6. Photolysis of diazoketone **10** in methanol.

[0049] After switching on the lamp, the 1 mM solution of compound **10** immediately starts to become red, and after 40 min no starting material could be detected in the reaction mixture. Two products have been isolated. Formation of the minor non-fluorescent product **11** may be explained by migration of the bond "a" in the intermediately formed carbene **10C.** The major red and fluorescent product **12** was formed in the course of Wolff rearrangement of carbene **10C** to ketene **10K** followed by addition of methanol to the highly reactive ketene group and breaking of the chemical bond with quaternary carbon atom. These results indicate that compounds of the general formula **I** may be effectively "uncaged", and their photolysis affords fluorescent rhodamine derivatives (e. g. compound **12**).



Scheme 7. Conversion of 6-carboxy tetramethylrhodamine **15** into bis(diazoketone) **17**: a) 85% aq. H_3PO_4, 170 °C, 6 h; b) $POCl_3$ in 1,2-dichloroethane, reflux, 4 h; CH_2N_2 in THF, 0 °C - room temp., 18 h; c) MeOH, CH_2Cl_2, $PhCO_2Ag$ (cat.), Et_3N, room temp., 8 h.

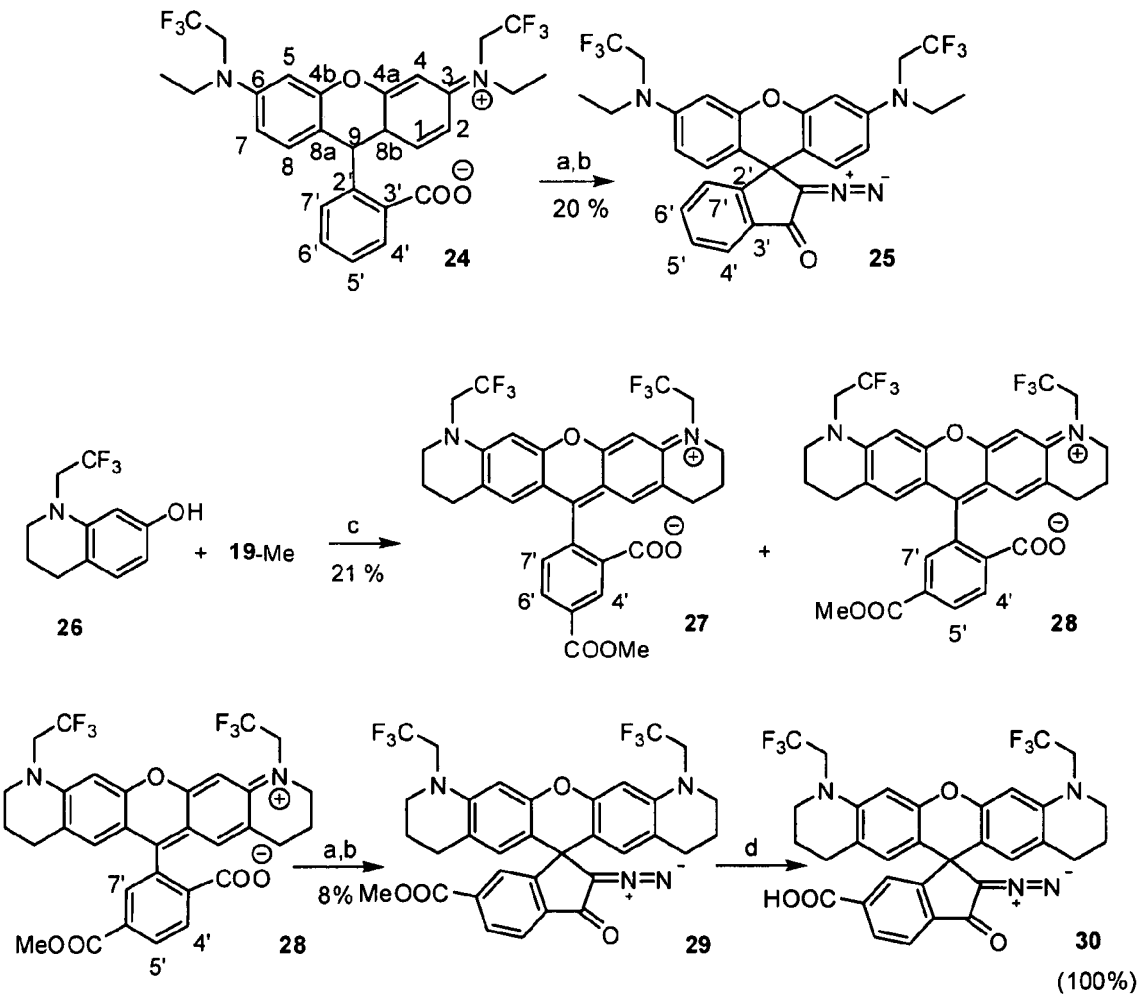
[0050] Practical use of the caged compounds **I** in bioconjugation procedures require the reagents with an additional functional group which may further be transformed into the amino or thiol reactive site. To provide compounds with an additional functional group in the benzoyl fragment, 5- and 6-carboxy tetramethylrhodamines (TMR) were used as starting materials (Scheme 7). Separation of a mixture of 5- and 6-carboxy TMR afforded the 6-carboxy derivative which was converted into the bis(diazoketone) **17.** Catalytic reaction in methanol in the presence of silver benzoate afforded mono diazoketone **18** with the intact caged fluorophore. Thus, it was possible selectively to transform one diazoketone group into the methyl ester, from which the corresponding *N*-hydroxysuccimidyl ester could easily be prepared according well-established protocols. Unfortunately, the overall yield of the ester **18** was low, and therefore it was necessary to improve the approach to the caged compounds **I** with an additional carboxy group.



Scheme 8. Preparation of the monoesters of 5(6)-carboxy tetramethylrhodamine and their conversion into diazoketones **22**-R and **23**-R: a) 1,2-dichlorobenzene, 170-190 °C, 19 h; b) $(COCl)_2$ in CH_2Cl_2, room temp., 2-4 h; 2 M Me_3SiCHN_2 in ether, CH_2Cl_2, Et_3N, 0 °C - room temp., 11-18 h; c) 1 M aq. NaOH, EtOH, THF, room temp., 16 h; d) *N,N,N',N'*-tetramethyl-*O*-(*N*-succinimidyl)isourea tetrafluoroborate, DMF, iPr_2NEt, room temp., 2 h.

[0051] The general methodology given in Scheme 8 allows not only to obtain various esters with a caged rhodamine fragment, but also to saponificate them keeping the spiro diazoketone fragment intact. If necessary, isomeric esters **20**(**22**-R) and **21**(**23**-R), as well as acids **22**-H and **23**-H, may be separated, but this procedure is tedious and in most of the practical applications mixtures of 5- and 6- isomers may be applied. For example, the mixture of *N*-hydroxysuccinimidyl esters **22**/**23**-NHS was used in immunostaining experiments.

[0052] Other rhodamines may be caged using the methodology of the present invention. For example, Schemes 9 and 10 illustrate the scope and some limitations associated with the new chemistry introduced here.

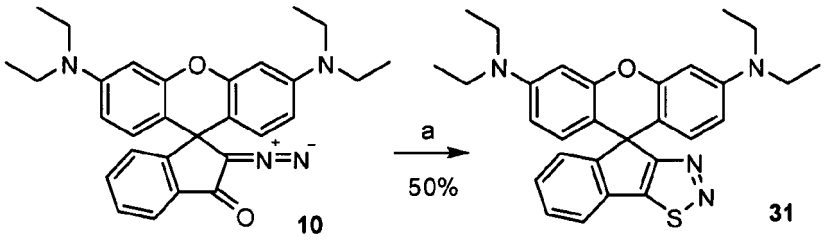


Scheme 9. Synthesis of the caged fluorescent dyes - spectral analogues of rhodamine 6G and rhodamine Q: a) $(COCl)_2$ in CH_2Cl_2, room temp., 0.5-2 h; b) 2 M $Me_3SiCH=N=N$ in ether, CH_2Cl_2, Et_3N, 0 °C - room temp., 11-14 h; c) 1,2-dichlorobenzene, 200 °C, 14 h; d) 1 M aq. NaOH, THF, room temp., 16 h.

[0053] To exemplify the possibility of multicolour staining with the new caged fluorescent dyes, spectral analogues of rhodamine 6G and rhodamine Q were prepared (Scheme 9). The electronic effects of the CF_3CH_2 substituents are similar to these of hydrogen atoms. Therefore, compound **24** has spectral properties (emission and absorption maxima) similar to these of rhodamine 6G (abs./em. at ca. 530/550 nm in MeOH), and rhodamines **27/28** are in this respect the analogues of 5/6-carboxy rhodamine Q (abs./em. at ca. 540/560 nm in MeOH). These values differ from each other and from the spectral positions of the absorption and emission bands found for rhodamine B, TMR and compound **12,** all of which absorb at about 560 nm and emit at ca. 580 nm (in MeOH).

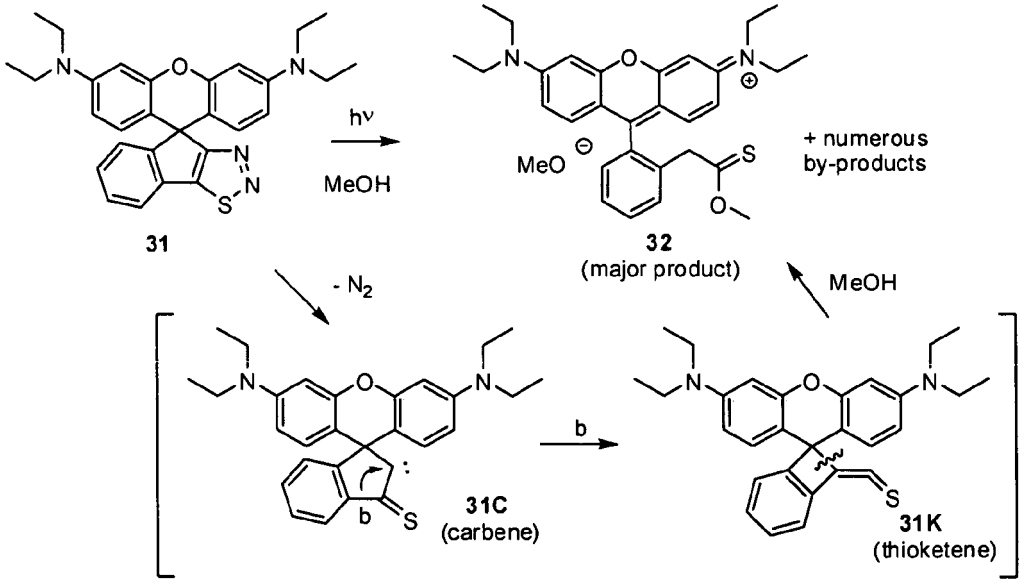
[0054] Modern microscopes (e. g. Zeiss META system) are able to resolve the differences between the fluorescence maxima, which are only 5-10 nm apart. Using these devices, a tool-box of at least two (caged!) fluorescent colours for multicolour labelling may be created by using for instance, compounds **22/23** for one colour and compound **30** for the other colour. After uncaging with a diode laser irradiating at 375 nm, the fluorescence of both dyes may be excited by the same light source (e. g. at 532 nm). In principle, this approach enables to observe, (co)localize and monitor in time the position of two objects labelled with the caged compounds **22/23** and **30,** respectively.

[0055] Preparation of the compound class **II** from the compound class **I** is exemplified in Scheme 10.



Scheme 10. Synthesis of 1,2,3-thiadiazole **31** (compound class **II**) from 2-diazoketone **10** (compound class **I**): a) Lawesson reagent, benzene, reflux, 1 h.

[0056] Photolysis of 1,2,3-thiadiazole **31** in methanol was found to be not as clean reaction as the photolysis of diazoketone **10** (Scheme 6). However, as the main product it afforded the colored and fluorescent thioester **32.**



Scheme 11. Photolysis of 1,2,3-thiadiazole **31** in methanol.

[0057] The invention is further illustrated by the following nonlimiting Examples and Figures.

FIGURES

[0058]

Fig. 1. Switching of compounds **22/23** in living and fixed eukaryotic cells. (A) Compounds **22/23** switched in living Ptk2 cells. (B) Compounds **22/23**-NHS coupled to antibodies, introduced into fixed cells and switched in Mowiol.

Fig. 2. Intensity-dependent switching kinetics of compounds **22/23.** (A) Kinetics under low light intensities, (B) Kinetics under higher light intensities.

Fig. 3. Tracking of proteins and organelles within living cells (A) images for t = 0 (before switching), t = 30, and t = 360 s; (B) Close-up of t= 363 s to t = 390 s.

Fig. 4. Conventional microscopic image (A) and single molecule localization microscopic (SMLM) image (B) of the microtubule network in a whole fixed PtK2 cell immunolabelled with secondary antibodies conjugated with compounds **22/23-**NHS.

Instruments and general parameters of the detection methods used

[0059] NMR spectra were recorded with Varian MERCURY 300 spectrometer at 300 (^{1}H) and 75.5 MHz (^{13}C and APT). All spectra are referenced to tetramethylsilane as an internal standard (δ = 0 ppm) using the signals of the residual protons of $CHCl_3$ (7.26 ppm) in $CDCl_3$, CHD_2OD (3.31 ppm) in CD_3OD, HOD (4.79 ppm) in D_2O or [D_5] DMSO (2.50 ppm) in [D_6] DMSO. Multiplicities of signals are described as follows:

s = singlet, br. s = broad singlet, d = doublet, br. d = broad doublet, t = triplet, q = quartet, m = multiplet. Coupling constants (*J*) are given in Hz. Multiplicities in the ^{13}C NMR spectra were determined by APT (Attached Proton Test) measurements. Low resolution mass spectra (electro spray ionization, ESI) were obtained with LCQ spectrometer. High resolution mass spectra (ESI-HRMS) were obtained on APEX IV spectrometer. HPLC system (Knauer): Smartline pump 1000 (2x), UV detector 2500, column thermostat 4000, mixing chamber, injection valve with 20 and 100 μL loop for the analytical and preparative columns, respectively; 6-port-3-channel switching valve; analytical column: Eurospher-100 C18, 5 μm, 250x4 mm, 1 mL/min; solvent A: water + 0.1% v/v trifluoroacetic acid (TFA) ; solvent B: MeCN + 0.1% v/v TFA; A/B: 60/40 $\rightarrow$ 0/100 in 25 min (unless stated otherwise); 25 °C, detection at 254 nm. Analytical TLC was performed on MERCK ready-to-use plates with silica gel 60 (F_{254}). Column chromatography: MERCK silica gel, grade 60, 0.04-0.063 mm. Elemental analyses were carried out at Mikroanalytisches Laboratorium des Instituts für Organische und Biomolekulare Chemie der Georg-August-Universität Göttingen. Organic solutions were dried over $MgSO_4$ or Na_2SO_4. Preparative photolysis reactions were performed with middle-pressure mercury lamp (150 W) with boron-silica glass filter (ca. 50% transmission at 320 nm) using the laboratory reactor UV-RS-1 manufactured by UV-Consulting Peschl (Mainz, Germany). The following commercially available microscopes were used for the (diffraction limited) uncaging experimets with antibodies labelled with NHS-ester(s) **22/23-**NHS: 1) epifluorescence microscope Leica DM6000, 2) confocal microscope Leica TCS SP5. Single molecule switching (SMS) experiments were performed using the PALMIRA setup [A. Egner, C. Geisler, C. von Middendorff, H. Bock, D. Wenzel, R. Medda, M. Andresen, A. C. Stiel, S. Jakobs, C. Eggeling, A. Schönle, S. W. Hell, Biophys. J. 2007, 93, 3285-3290] equipped with 532 nm solid state laser and an additional optical path for the diode laser operating at 375 nm.

EXAMPLE 1

*Synthesis and photolysis of specific compounds of formulae **I-III***

[0060] **Diazomethane.** Diazomethane should be handled with care since it is known to be carcinogenic and may explode on heating. Erlenmeyer (conical) flasks may be used for its synthesis. Diazomethane was prepared in THF (150 mL, without peroxides) using *N*-nitroso-*N*-methylurea (NMU, 7.3 g) and 40% aq. KOH solution (100 g) at 0°-5°C. External cooling with an ice or ice-salt bath should be used. NMU was carefully introduced in small portions with a plastic spatula into the two-phase system consisting of THF and aq. KOH at 0-5°C with a temperature control inside the flask. *No mechanical stirring!* The reaction mixture may be shaken or stirred by hand with another plastic spatula. (Only new flasks and thermometers without ground joints and scratches should be used). After the reaction was complete, the yellow organic solution was carefully decanted from the lower aqueous layer to the new flask with solid KOH. After drying for 30 min at 0°C, the solution was used for the synthesis of diazoketones. The freshly prepared diazomethane solution may be kept at -20°C for several days.

[0061] **Diazoketone 10:** Rhodamine B (1.1 g, 2.3 mmol) and 1,2-dichloroethane (DCE, 11 mL) were placed into a dry 250 mL Schlenk flask. $POCl_3$ (0.9 mL, 9.7 mmol) was added, and the mixture was refluxed for 4 h. Then the solvent and excess of $POCl_3$ were removed in vacuo. The residue was dissolved with THF (10 mL), and a solution of CH_2N_2 (ca. 72 mmol) in THF (150 mL) was added to the reaction mixture at 0°C with stirring. The reaction mixture was kept for 12 h at room temperature. Then the solvent and excess of CH_2N_2 were removed in vacuo [outlet of the vacuum pump has to be inside a well-ventilated hood], and the title product was isolated from the residue by column chromatography (CH_2Cl_2). The pure title compound was obtained after crystallization from the mixture of CH_2Cl_2 and MeOH (1:2); yield 0.77 g (72%) ; m. p. 194°C (dec.). HPLC: t_R = 2.5 min; HPLC (conditions: A/B: 70/30 $\rightarrow$ 0/100 in 25 min) : t_R = 7.8 min. UV (MeCN): λ_{max} = 241 nm (ε = 59700), 268 nm (sh.), 312 nm (sh). ^{1}H NMR ($CDCl_3$, 300 MHz, ppm), δ = 1.15 (t, *J* = 7.1, 12 H; $(CH_2)CH_3$), 3.31 (q, *J* = 7.1, 8 H; $NCH_2(CH_3)$), 6.28 (dd, *J* = 2.7 and 8.8, 2H; H-2/7), 6.37 (d, *J* = 2.6, 2 H; H-4/5), 6.67 (d, *J* = 8.8, 2 H; H-1/8), 7.08 (br. d, *J* = 7.6, 1 H; H-7'), 7.34-7.40 (m, 1 H; H-6'), 7.42-7.48 (m, 1 H; H-5'), 7.80 (br. d, *J* = 6.8, 1 H; H-4'). ^{13}C NMR ($CDCl_3$, 75.5 MHz, ppm), δ = 12.6 ((CH_2) *C*H_3), 44.3 (NCH_2 (CH_3)), 49.1 (C; C-1'), 76.8 (C; *C*N_2), 98.1 (CH; C-4/5), 107.6 (C; C-8a/8b), 108.0 (CH; C-2/7), 122.0 (CH; C-7'), 125.5 (CH; C-4'), 128.1 (CH; C-5'),128.7 (CH; C-1/8), 134.46 (CH; C-6'), 134.53 (C; C-2'), 148.3 (C; C-3/6), 152.5 (C; C-4a/4b), 156.4 (C; C-3'), 187.5 (CO). ESI-MS, positive mode: *m*/*z* (rel. int., %) = 489 (31) [*M*+Na]$^+$, 955 (100) [2*M*+Na]$^+$. EI-MS: *m*/*z* (rel. int., %) = 466 [*M*]$^+$ (20), 438 [*M*-N_2]$^+$ (100), 423 [*M*-N_2-CH_3]$^+$ (35). $C_{29}H_{30}N_4O_2$, HR-MS (ESI, positive mode): 467.2430 [*M*+H]$^+$ (found),

467.2442 (calculated).

[0062] **Photolysis of diazoketone 10:** A solution of compound **10** (70 mg, 0.15 mmol) in MeOH (150 mL) was placed into a reactor for photolysis, and nitrogen was bubbled for 30 min through the solution at room temperature with stirring. Then the lamp was turned on, and the reaction mixture was irradiated at ambient temperature with stirring under N_2 for 40 min, until the starting compound could not be detected on TLC. The solvent was removed in vacuo. The residue was dissolved in CH_2Cl_2, and the two products were isolated by column chromatography (CH_2Cl_2, then CH_2Cl_2/MeOH 5:1).

[0063] Compound **11** with a higher R_f-value eluted first, yielding 11 mg (17%) of a dark solid. HPLC: t_R = 8.9 min. UV (MeOH) : λ_{max} = 574 nm (ε = 3980). ^{1}H NMR ($CDCl_3$, 300 MHz, ppm), δ = 1.17 (t, *J* = 7.1, 6 H; (CH_2) CH_3), 1.20 (t, *J* = 7.1, 6 H; (CH_2) CH_3) , 3.36 (q, *J* = 7.0, 4 H; NCH_2(CH_3)), 3.40 (q, *J* = 7.0, 4 H; NCH_2(CH_3)), 6.49-6.53 (m, 2 H; H-2/7), 6.54 (d, *J* = 2.1, 1 H; H-4(5)), 6.58 (d, *J* = 2.7, 1 H; H-5(4)), 7.15-7.23 (m, 1 H; H-4'), 7.28-7.34 (m, 1 H; H-5'), 7.39 (br. d, *J* = 7.2, 1 H; H-6'), 7.50 (br. d, *J* = 7.0, 1 H; H-3'), 7.58 (d, *J* = 8.8, 1 H; H-8), 7.77 (d, *J* = 9.3, 1 H; H-1). ^{13}C NMR ($CDCl_3$, 75.5 MHz, ppm), δ=12. 55 ((CH_2) *C*H_3), 12.57 ((CH_2) *C*H_3), 44.5 (N*C*H_2 (CH3)), 44.6 (NCH_2(CH_3)), 103.6 and 104.5 (2CH; C-4/5), 107.8 and 108.6 (2CH; C-2/7), 112.8 and 114.1 (2CH; C-8a/8b), 120.8 (CH; C-6'), 122.3 (CH; C-3'), 126.9 (C; C-1'), 127.9 (CH; C-4'), 128.3 (CH; C-8), 129.8 (CH; C-1), 132.7 (CH; C-5'), 144.2 (C; C-6a'), 149.5 (C; C-3), 151.5 (C; C-6), 158.4 (C; C-4a), 159.8 (C; C-4b), 195.8 (CO). ESI-MS, positive mode: *m*/*z* (rel. int., %) = 461 (33) [*M*+Na]$^+$, 439 (100) [*M*+H]$^+$, 438 (100) [*M*]$^+$,. EI-MS: *m*/*z* (rel. int., %) = 438 [*M*]$^+$ (100), 423 [*M*-CH_3]$^+$ (28). DCI-MS: *m*/*z* (rel. int., %) = 439 (100) [*M*+H]$^+$. $C_{29}H_{30}N_2O_2$, HR-MS (ESI, positive mode) : 438.23015 [*M*]$^+$ (found), 439.23018 (calculated); 439.23796 [*M*+H]$^+$ (found), 438.23800 (calculated).

[0064] Compound **12,** which eluted after that, was isolated as a dark red solid; yield 25 mg (35%); HPLC: t_R = 8.8 min. UV (MeOH): λ_{max} = 559 nm (ε = 65600), λ_{em} = 579 nm, Φ_{fl} = 0.37. ^{1}H NMR ($CDCl_3$, 300 MHz, ppm), δ = 1.29 (t, *J* = 7.1, 12 H; (CH_2) CH_3), 3.35 (s, 2 H; C$H_2CO_2CH_3$) , 3.39 (s, 3 H; CO_2CH_3) , 3.63 (q, *J* = 7.1, 8 H; NCH_2(CH_3)), 6.82 (d, *J* = 2.3, 2 H; H-4/5), 6.88 (dd, *J* = 2.4 and 9.5, 2 H; H-2/7), 7.10 (d, *J* = 9.5, 2 H; H-1/8), 7.16 (dd, *J* = 1.1 and 7.1, 1 H; H-7'), 7.42-7.60 (m, 3 H; H-4'/5'/6'). ^{13}C NMR ($CDCl_3$, 75.5 MHz, ppm), δ = 12.6 ((CH_2)CH_3), 29.6 (($CH_2CO_2CH_3$), 38.6 (NCH_2 (CH_3)), 52.0 (OCH_3) , 96.5 (CH; C-4/5), 113.6 (C; C-8a/8b), 114.3 (CH; C-2/7), 127.8 (CH), 129.3 (CH), 130.4 (CH), 131.2 (CH), 131.7 (CH; C-1/8), 131.9 (C), 132.4 (C), 155.7 (C; C-3/6), 157.8 (C; C-4a/4b), 156.0 (C), 170.7 (CO_2CH_3)). ESI-MS, positive mode: *m*/*z* (rel. int., %) = 471 (100) [*M*+H]$^+$. $C_{30}H_{35}N_2O_3$ (+), HR-MS (ESI, positive mode): 471.2629 [*M*]$^+$ (found), 471.2642 (calculated).

[0065] **Mixture of TMR 5/6-carboxylates (15 + 16):** A mixture of finely grounded 3-(*N*,*N*-dimethylamino)phenol (2.6 g, 19 mmol) and trimellitic anhydride (4.8 g, 26 mmol) was heated at 170 °C for 3 h. An additional portion of 3-(*N*,*N*-dimethylamino) phenol (2.6 g, 19 mmol) and 85% aq. H_3PO_4 (7 mL) were added to the cooled reaction mixture, and heating was continued at 170°C for 3 h. After cooling, the reaction mixture was stirred and refluxed with a mixture of methanol (150 mL) and water (50 mL) for several minutes, cooled, and kept at room temperature for 10 h. The precipitate was filtered off, yielding a mixture of compounds **15** (major diastereomer, HPLC: t_R = 8.2 min) and **16** (minor diastereomer, HPLC: t_R = 9.2 min); 0.94 g (12%). ESI-MS, positive mode: *m*/*z* (rel. int., %) = 431 (100) [*M*+H]$^+$. ESI-MS, negative mode: *m*/*z* (rel. int., %) = 429 (100) [*M*-H]$^-$.

[0066] The product was recrystallized from methanol and the isomer **15** (0.64 g, 8%) with content of the major diastereomer of about 90% was isolated.

[0067] **Bis-diazoketone 17:** Crude rhodamine **15** (0.11 g, 0.25 mmol) and DCE (1.0 mL) were placed into a dry 50 mL Schlenk flask under N_2. $POCl_3$ (0.6 mL, 6.4 mmol) was added to the flask. The mixture was refluxed for 3.5 h. Then the solvent and excess of $POCl_3$ were removed in vacuo. The residue was dissolved with THF (5 mL), and a solution of CH_2N_2 (10 mmol) in THF (30 mL) was added to the reaction mixture at 0 °C with stirring. The reaction mixture was kept at room temperature for 18 h. The solvent and excess of CH_2N_2 were removed in vacuo [outlet of the vacuum pump has to be inside a well-ventilated hood], and the title product was isolated from the residue by column chromatography (CH_2Cl_2 / EtOAc (10:1)); yield 25 mg (21%). HPLC: t_R = 10.9 min. ^{1}H NMR ($CDCl_3$, 300 MHz, ppm), δ = 2.94 (s, 12 H; NCH_3) , 5.76 (s, 1 H; $COCHN_2$), 6.35 (dd, *J* = 2.6 and 8.8, 2 H; H-2/7), 6.43 (d, *J* = 2.6, 2 H; H-4/5), 6.68 (d, *J* = 8.8, 2 H; H-1/8), 7.36 (d, *J* = 0.8, 1 H; H-7'), 7.77 (dd, *J* = 1.5 and 8.0, 1 H; H-5'), 7.86 (d, *J* = 8.0, 1 H; H-4'). ^{13}C NMR ($CDCl_3$, 75.5 MHz, ppm), δ = 40.3 (NCH_3) , 49.2 (C; C-1'), 55.1 (CH; CO*C*HN_2), 78.1 (C; *C*N_2), 99.3 (CH; C-4/5), 107.6 (C; C-8a/8b), 108.9 (CH; C-2/7), 122.5 (CH; C-4'), 123.7 (CH; C-7'), 126.8 (CH; C-5'), 128.4 (CH; C-1/8), 137.9 (C; C-3'), 141.8 (C; C-6'), 151.1 (C; C-3/6), 152.1 (C; C-4a/4b), 156.5 (C; C-2'), 185.1 ($COCHN_2$), 185.9 (CO). ESI-MS, positive mode: *m*/*z* (rel. int., %) = 501 (14) [M+Na]$^+$, 979 (100) [2*M*+Na]$^+$; found, %: C 67.90, H 4.90, N 17.30; anal. calcd. (%) for $C_{27}H_{22}N_6O_3$ (478.50): C 67.77, H 4.63, N 17.56.

[0068] **Mono-diazoketone 18:** Bis-diazoketone **17** (0.11 g, 0.25 mmol) was dissolved in a mixture of CH_2Cl_2 (1 mL) and MeOH (1mL). A suspension of $PhCO_2Ag$ (10 mg, 0.05 mmol) and Et_3N (0.2 mL, 1.5 mmol) in MeOH (0.8 mL) was added to the reaction mixture at room temperature. The mixture was stirred at room temperature for 8 h, and then filtered through a plug of SiO_2. The solvent was removed in vacuo, and the title product was isolated from the residue by column chromatography (CH_2Cl_2 / EtOAc (4:1)); yield 20 mg (18%). HPLC: t_R = 10.7 min. ^{1}H NMR ($CDCl_3$, 300 MHz, ppm), δ = 2.95 (s, 12 H; NCH_3) , 3.53 (s, 2 H; Ar$CH_2CO_2CH_3$) , 3.60 (s, 1 H; Ar$CH_2CO_2CH_3$) , 6.36 (dd, *J* = 2.5 and 8.7, 2 H; H-2/7), 6.42 (d, *J* = 2.5, 2 H; H-4/5), 6.71 (d, *J*= 8.7, 2 H; H-1/8), 6.90 (br. s, 1 H; H-7'), 7.35 (br. d, *J* = 7.9, 1 H; H-5'), 7.77

(d, J = 7.9, 1 H; H-4'). ^{13}C NMR ($CDCl_3$, 75.5 MHz, ppm) , δ = 40.3 (NCH_3) , 41.1 (CH; $ArCH_2CO_2CH_3$) , 48.9 (C; C-1'), 52.1 (OCH_3) , 77.1 (C; CN_2), 99.1 (CH; C-4/5), 108.5 (C; C-8a/8b), 108.8 (CH; C-2/7), 122.3 (CH), 126.3 (CH), 128.6 (CH; C-1/8), 129.6 (CH), 133.6 (C), 140.8 (C), 151.0 (C; C-3/6), 152.1 (C; C-4a/4b), 156.6 (C), 171.0 (CO_2CH_3), 186.8 (CO). ESI-MS, positive mode: m/z (rel. int., %) = 505 (41) $[M+Na]^+$, 987 (100) $[2M+Na]^+$. ESI-MS, negative mode: m/z (rel. int., %) = 481 (100) $[M-H]^-$. $C_{29}H_{26}N_4O_4$, HR-MS (ESI, positive mode): 483.20271 $[M+H]^+$ (found), 483.20268 (calculated).

[0069] **Esters 20-Et + 21-Et:** A mixture of 3-(*N,N*-dimethylamino)phenol (1.65 g, 12 mmol), trimellitic anhydride monoethyl ester **19-Et** (3.1 g, 14 mmol), and 1,2-dichlorobenzene (12 mL) was heated at 190 °C for 5 min with stirring under N_2. Then an additional portion of 3-(*N,N*-dimethylamino)phenol (1.65 g, 12 mmol) was added to the cooled reaction mixture and heating was continued at 190 °C for 19 h. Then the solvent was removed in vacuo, and the title product was isolated from the residue by column chromatography (CH_2Cl_2 / MeOH (2:1)) as a mixture of two regioisomers (2 : 1; according to ^{1}H NMR data); yield - 2.9 g (53%). HPLC: t_R = 11.3 min (for both inseparable isomers). ESI-MS, positive mode: m/z (rel. int., %) = 459 (100) $[M+H]^+$, 481 (8) $[M+Na]^+$.

[0070] The major isomer **21-Et:** ^{1}H NMR (CD_3OD, 300 MHz, ppm), δ = 1.44 (t, J = 7.2, 3 H; $(CH_2)CH_3$), 3.25 (s, 12 H; NCH_3) , 4.45 (q, J = 7.2, 2 H; $OCH_2(CH_3)$), 6.86-6.90 (m, 2 H; H-4/5), 6.94-7.02 (m, 2 H; H-2/7), 7.21 (d, J = 9.5, 2 H; H-1/8), 7.36 (d, J = 7.9, 1 H; H-7'), 8.21 (dd, J = 1.8 and 7.9, 1 H; H-6'), 8.74 (d, J = 1.5, 1 H; H-4').

[0071] The minor isomer **20-Et:** ^{1}H NMR (CD_3OD, 300 MHz, ppm) , δ = 1.36 (t, J = 7.1, 3 H; (CH_2) CH_3) , 3.25 (s, 12 H; NCH_3) , 4.36 (q, J = 7.1, 2 H; OCH_2 (CH_3)), 6.86-6.90 (m, 2 H; H-4/5), 6.94-7.02 (m, 2 H; H-2/7), 7.22 (d, J = 9.5, 2 H; H-1/8), 7.85 (d, J = 1.3, 1 H; H-7'), 8.13 (d, J = 8.1, 1 H; H-4'), 8.26 (dd, J = 1.7 and 8.1, 1 H; H-5').

[0072] **Diazoketones 22-Et + 23-Et:** A mixture of regioisomers **20-Et + 21-Et** (0.94 g, 2.0 mmol) was dissolved with CH_2Cl_2 (20 mL),and oxalyl chloride ($(COCl)_2$, 0.9 mL, 11 mmol) was added to the flask. The mixture was kept at room temperature for 4 h. Then the solvent and excess of $(COCl)_2$ were removed in vacuo. The residue was dissolved with CH_2Cl_2 (20 mL); Et_3N (0.25 mL) and a solution of Me_3SiCHN_2 in Et_2O (2 mL of 2 M solution, 4 mmol) were added to the reaction mixture at 0 °C with stirring. The reaction mixture was kept at room temperature for 18 h. Then the solvent and excess of Me_3SiCHN_2 were removed in vacuo (hood!), and the title product was isolated from the residue by column chromatography (CH_2Cl_2 / EtOAc (10:1)), yielding 0.77 g (80%) of a crude mixture of the regioisomers. UV (MeCN): λ_{max} = 232 nm (ε = 74300), 263 nm (sh), 306 nm (sh). ESI-MS, positive mode: m/z (rel. int., %) = 505 (16) $[M+Na]^+$, 537 (100) $[M+Na+MeOH]^+$, 979 (70) $[2M+Na]^+$; found, %: C 69.75, H 5.24, N 11.47; anal. calcd. (%) for $C_{28}H_{26}N_4O_4$ (482.20): C 69.70, H 5.43, N 11.61.

[0073] The major isomer **23-Et:** HPLC: t_R = 16.2 min. ^{1}H NMR ($CDCl_3$, 300 MHz, ppm), δ = 1.37 (t, J = 7.2, 3 H; (CH_2) CH_3), 2.95 (s, 12 H; NCH_3) , 4.37 (q, J = 7.2, 2 H; OCH_2 (CH3)), 6.35 (dd, J = 2.6 and 8.7, 2 H; H-2/7), 6.43 (d, J = 2.5, 2 H; H-4/5), 6.69 (d, J = 8.7, 2 H; H-1/8), 7.10 (d, J = 8.1, 1 H; H-7'), 8.11 (dd, J = 1.6 and 8.1, 1 H; H-6'), 8.47 (d, J = 1.2, 1 H; H-4'). ^{13}C NMR ($CDCl_3$, 75.5 MHz, ppm), δ = 14.3 (CH_2CH_3), 40.3 (NCH_3), 49.2 (C; C-1'), 61.3 (OCH_2CH_3), 77.1 (CN_2), 99.2 (CH; C-4/5), 107.7 (C; C-8a/8b), 108.9 (CH; C-2/7), 123.7 (CH), 125.4 (CH), 128.5 (CH; C-1/8), 130.8 (C), 134.7 (C), 135.5 (CH), 151.1 (C; C-3/6), 152.1 (C; C-4a/4b), 160.1 (C), 165.6 (CO_2CH_3) , 186.2 (CO).

[0074] The minor isomer **22-Et**: HPLC: t_R = 15.3 min. ^{1}H NMR ($CDCl_3$, 300 MHz, ppm), δ = 1.29 (t, J= 7.2, 3 H; (CH_2) CH_3), 2.95 (s, 12 H; NCH_3) , 4.26 (q, J = 7.2, 2 H; OCH_2 (CH3)), 6.35 (dd, J = 2.6 and 8.8, 2H; H-2/7), 6.44 (d, J = 2.7, 2H; H-4/5), 6.68 (d, J = 8.7, 2 H; H-1/8), 7.68 (br. s, 1 H; H-7'), 7.86 (dd, J = 0.6 and 8.0, 1 H; H-4'), 8.06 (dd, J = 1.4 and 8.0, 1 H; H-5'). ^{13}C NMR ($CDCl_3$, 75.5 MHz, ppm), δ = 14.2 (CH_2CH_3) , 40.3 (NCH_3) , 49.1 (C; C-1'), 61.4 (OCH_2CH_3) , 77.2 (CN_2), 99.3 (CH; C-4/5), 107.7 (C; C-8a/8b), 108.9 (CH; C-2/7), 122.1 (CH), 126.7 (CH), 128.5 (CH; C-1/8), 129.6 (C), 136.0 (C), 138.2 (CH), 151.1 (C; C-3/6), 152.2 (C; C-4a/4b), 156.0 (C), 165.6 (CO_2CH_3), 186.1 (CO).

[0075] **Esters 20-Me + 21-Me:** A mixture of 3-(*N,N*-dimethylamino)phenol (3.3 g, 24 mmol), trimellitic anhydride monomethyl ester **19-Me** (3.45 g, 17 mmol), and 1,2-dichlorobenzene (20 mL) was heated at 190 °C with stirring under N_2 for 5.5 h. Then the solvent was removed in vacuo, and the title product was isolated from the residue by column chromatography (CH_2Cl_2 / MeOH (2:1)), yielding 3.1 g (58%) of a mixture of two regioisomers. ESI-MS, positive mode: m/z (rel. int., %) = 445 (100) $[M+H]^+$, 467 (5) $[M+Na]^+$.

[0076] **21-Me:** HPLC: t_R = 9.8 min. ^{1}H NMR (CD_3OD, 300 MHz, ppm) , δ = 3.23 (s, 12 H; NCH_3), 4.00 (s, 3 H; OCH_3), 6.80-6.85 (m, 2 H; H-4/5), 6.91-7.00 (m, 2 H; H-2/7), 7.20 (d, J = 9.4, 2 H; H-1/8), 7.37 (d, J = 7.9, 1 H; H-7'), 8.20 (dd, J = 1.7 and 7.9, 1 H; H-6'), 8.74 (d, J = 1.5, 1 H; H-4').

[0077] **20-Me:** HPLC: t_R = 9.5 min. ^{1}H NMR (CD_3OD, 300 MHz, ppm), δ = 3.22 (s, 12 H; NCH_3) , 3.87 (s, 3 H; OCH_3) , 6.80-6.85 (m, 2 H; H-4/5), 6.91-7.00 (m, 2 H; H-2/7), 7.19 (d, J = 9.4, 2 H; H-1/8), 7.85 (d, J = 1.6, 1 H; H-7'), 8.14 (d, J = 8.2, 1 H; H-4'), 8.24 (dd, J = 1.7 and 8.1, 1 H; H-5').

[0078] **Diazoketone 23-Me:** A mixture of regioisomers **20-Me + 21-Me** (1.53 g, 3.4 mmol) was dissolved with CH_2Cl_2 (30 mL), and $(COCl)_2$ (1.1 mL, 13 mmol) was added to the flask. The mixture was kept at room temperature for 2 h. Then the solvent and excess of $(COCl)_2$ were removed in vacuo. The residue was dissolved in CH_2Cl_2 (30 mL). Et_3N (0.60 mL, 4.2 mmol) and a solution of Me_3SiCHN_2 in Et_2O (3 mL of 2 M solution, 6 mmol) were added to the reaction mixture at room temperature with stirring. The reaction mixture was kept at room temperature for 11 h. Then the solvent and excess of Me_3SiCHN_2 were removed in vacuo, and the title product was isolated from the residue by column

chromatography (CH_2Cl_2 / EtOAc (20:1)), yield of the crude mixture of the regioisomers 0.92 g (57%). After crystallization from CH_2Cl_2/methanol (1:4), the pure isomer **23-Me** (0.72 g, 45%) was isolated; m. p. > 215°C (dec.). UV (MeCN): λ_{max} = 231 nm (= 82700), 263 nm (sh), 306 nm (sh). ESI-MS, positive mode: *m*/*z* (rel. int., %) = 523 (58) [*M*+Na+MeOH]$^+$, 959 (100) [2*M*+Na]$^+$, 1427 (35) [3*M*+Na]$^+$. Found, %: C 69.05, H 5.07, N 11.83; anal. calcd. (%) for $C_{27}H_{24}N_4O_4$ (468,50): C 69.22, H 5.16, N 11.96. HPLC: t_R = 14.2 min. ^{1}H NMR ($CDCl_3$, 300 MHz, ppm), δ = 2.94 (s, 12 H; NCH_3), 3. 91 (s, 3 H; OCH_3), 6.35 (dd, *J* = 2. 6 and 8.8, 2 H; H-2/7), 6.43 (d, *J* = 2.7, 2H; H-4/5), 6.70 (d, *J* = 8.7, 2 H; H-1/8), 7.10 (dd, *J* = 0.7 and 8.1, 1 H; H-7'), 8.11 (dd, *J* = 1.6 and 8.1, 1 H; H-6'), 8.47 (dd, *J* = 0.7 and 1. 7, 1 H; H-4'). ^{13}C NMR ($CDCl_3$, 75.5 MHz, ppm), δ = 40.3 (NCH_3), 49.1 (C; C-1'), 52.4 (OCH_3), 77.1 (CN_2), 99.2 (CH; C-4/5), 107.7 (C; C-8a/8b), 108.9 (CH; C-2/7), 123.8 (CH), 125.5 (CH), 128.4 (CH; C-1/8), 130.5 (C), 134.7 (C), 135.5 (CH), 151.1 (C; C-3/6), 152.1 (C; C-4a/4b), 160.2 (C), 166.1 (CO_2CH_3), 186.1 (CO). Compound **22-Me:** HPLC: t_R = 13.8 min.

[0079] Carboxylic acids 22-H + 23-H: A mixture of the regioisomers **22-Et + 23-Et** (0.37 g, 0.77 mmol) was dissolved in THF (20 mL) and EtOH (50 mL) under N_2, and 1 M aq. NaOH (5 mL) was added. The reaction mixture was kept at room temperature for 16 h. Volatile solvents were evaporated in vacuo, the residue was dissolved in H_2O (20 mL), and acidified with 1 M aq. HCl to pH=6. The title compounds were extracted with CH_2Cl_2. The organic layer was dried with Na_2SO_4, the solvent was removed in vacuo, and the product was purified by column chromatography (CH_2Cl_2 / MeOH (5:1)) yielding the mixture of the regioisomers **22**-H and **23**-H; 0.32 g (92%). ESI-MS, positive mode: *m*/*z* (rel. int., %) = 455 (23) [*M*+H]$^+$, 909 (10) [2*M*+H]$^+$, 931 (100) [2*M*+Na]$^+$. ESI-MS, negative mode: *m*/*z* (rel. int., %) = 453 (96) [*M*-H]$^-$, 907 (100) [2*M*-H]$^-$, 1361 (14) [3*M*-H]$^-$.

[0080] A sample (0.10 g) was crystallized from CH_2Cl_2/methanol mixture (1:4) and gave the pure acid **22-H.** Found, %: C 68.91, H 4.74, N 12.10; calcd. (%) for $C_{26}H_{22}N_4O_4$ (454,48): C 68.71, H 4.88, N 12.33. HPLC: t_R = 7.8 min. UV (MeOH): λ_{max} = 231 nm (ε = 64000), 262 nm (sh), 307 nm (ε = 19000). ^{1}H NMR ($CDCl_3$, 300 MHz, ppm), δ = 2.93 (s, 12 H; NCH_3), 6.36 (dd, *J* = 2.6 and 8.8, 2H; H-2/7), 6.43 (d, *J* = 2.5, 2 H; H-4/5), 6.68 (d, *J* = 8.7, 2 H; H-1/8), 7.72 (br. s, 1 H; H-7'), 7.87 (d, *J* = 8.0, 1 H; H-4'), 8.08 (dd, *J* = 1.2 and 8.0, 1 H ; H-5'). ^{13}C NMR ($CDCl_3$, 75.5 MHz, ppm), δ = 40.3 (NcH_3), 49.4 (C; C-1'), 78.2 (C; *C*N_2), 99.7 (CH; C-4/5), 107.9 (C; C-8a/8b), 109.2 (CH; C-2/7), 122.3 (CH), 127.3 (CH), 128.3 (CH; C-1/8), 130.1 (CH), 135.0 (C), 138.8 (C), 151.3 (C; C-3/6), 152.3 (C; C-4a/4b), 156.2 (C), 169.7 (*C*O_2H), 186.1 (CO).

Compound **23-H,** HPLC: t_R = 8.8 min.

[0081] NHS-Esters 22/23-NHS were prepared from **22/23**-H with a ratio of **22**-H/**23**-H = ca. 2:3. Compound **22/23**-H (130 mg, 0.28 mmol) and *N,N,N',N'*-tetramethyl-*O*-(*N*-succinimidyl)isourea tetrafluoroborate (0.15 g, 0. 5 mmol) were dissolved in dry DMF (2 mL), and *i*Pr_2NEt (0.2 mL) was added. The reaction mixture became dark. After stirring for 2 h at room temperature, the solvent was evaporated in vacuo (1 mbar), and the residue was dissolved in small amount of CH_2Cl_2 and applied onto a pad of SiO_2 (25 g). Elution with EtOAc followed by evaporation of the yellow eluate afforded the title compounds (0.125 g, 81%) as a yellow foam which crystallized by triturating with hexane - EtOAc mixture. ^{1}H NMR ([D_6] DMSO, 300 MHz, signals of the major isomer **23**-NHS are marked with *, ppm), δ = 2.92 (s, 16 H; NCH_3 and CH_2CH_2), 6.43 (dd, *J* = 2 and 9; H-2/7), 6.48 (d, *J* = 2, E 4 H; H-4/5), 6.78 (2xd, *J* = 9, 2 H; H-1/8), 7.29* (d, *J* = 8; H-7'), 7.58 (d, *J* = 2; H-7'), 8.02 (d, *J* = 8; H-4'), 8.08 (dd, *J* = 2 and 8; H-5'), 8.25* (dd, *J* = 2 and 8; H-6'), 8.32* (d, *J* = 2; H-4'); ESI-MS, positive mode: *m*/*z* (rel. int., %) = 574 (100) [*M*+Na]$^+$. HPLC: t_R = 11.2/12.2 min (ratio ca. 4:1 in the analytical sample after additional purification by chromatography and precipitation from CH_2Cl_2 with hexane)

[0082] Diazoketone 25: Rhodamine **24** (0.14 g, 0.25 mmol) was dissolved in CH_2Cl_2 (5 mL), and $(COCl)_2$ (0.4 mL, 3.5 mmol) was added to the flask. The mixture was kept at room temperature for 2 h. Then the solvent and excess of $(COCl)_2$ were removed in vacuo. The residue was dissolved in CH_2Cl_2 (5 mL); Et_3N (0.10 mL, 0.7 mmol) and a solution of Me_3SiCHN_2 in Et_2O (0.5 mL of 2 M solution, 1 mmol) were added at room temperature with stirring. The reaction mixture was kept at room temperature for 11 h. The solvent and excess of Me_3SiCHN_2 were removed in vacuo, and the title product was isolated from the residue by column chromatography (CH_2Cl_2 / EtOAc (10:1)). After crystallization from CH_2Cl_2/*n*-hexane mixture (1:3), the pure diazoketone **25** (28 mg, 20%) was obtained. ^{1}H NMR ($CDCl_3$, 300 MHz, ppm), δ = 1.19 (t, *J* = 7.1, 6 H; (CH_2) CH_3), 3.47 (q, *J* = 7.1, 4 H; NCH_2(CH_3)), 3.82 (q, *J* = 8.9, 4 H; NCH_2(CF_3)), 6.40 (dd, *J* = 2.7 and 8.8, 2 H; H-2/7), 6.52 (d, *J* = 2.7, 2 H; H-4/5), 6.73 (d, *J* = 8.8, 2 H; H-1/8), 7.05 (br. d, *J* = 7.4, 1 H; H-7'), 7.36 - 7.50 (m, 2 H; H-5' and H-6'), 7.82 (br. d, *J* = 7. 3, 1H; H-4'). ^{13}C NMR ($CDCl_3$, 75.5 MHz, ppm), δ = 11.5 ($(CH_2)CH_3$), 45.8 ($NCH_2(CH_3)$), 48.8 (C; C-1'), 52.2 (q, *J* = 33; N$CH_2(CF_3)$), 77.2 (C; *C*N_2), 100.3 (CH; C-4/5), 109.3 (CH; C-2/7), 110.6 (C; C-8a/8b), 122.3 (CH; C-7'), 125.4 (q, *J* = 281; CF_3) , 125.5 (CH; C-4'), 128.5 (CH; C-5'),128.9 (CH; C-1/8), 134.59 (CH; C-6'), 134.71 (C; C-2'), 148.1 (C; C-3/6), 152.3 (C; C-4a/4b), 155.8 (C; C-3'), 187.0 (CO). EI-MS: *m*/*z* (rel. int., %) = 574 [*M*]$^+$ (8), 546 [*M*-N_2]$^+$ (100), 531 [*M*-N_2-CH_3]$^+$ (4), 477 [*M*-N_2-CF_3]$^+$ (6).

[0083] Rhodamine 28: A mixture of compound **26** (0.60 g, 2.6 mmol), trimellitic anhydride monomethyl ester **19-Me** (0.60 g, 2.9 mmol), and 1,2-dichlorobenzene (3 mL) was heated at 200 °C with stirring under N_2 for 5 min. The reaction mixture was cooled, another portion of compound **26** (0.60 g, 2.6 mmol) was added, and the mixture was heated at 200 °C with stirring under N_2 for 14 h. Then the solvent was removed in vacuo, a mixture of CH_2Cl_2 (20 mL) and MeOH (20 mL) was added to the residue, and the title product **28** (with ca. 10 % of the minor diastereomer **27**) was filtered off. The yield was 0.35 g (21%); m. p. = 143-145 °C. An additional portion of the diastereomeric mixture (0.38 g, 23%) precipitated

from the filtrate in one day. ^{1}H NMR ($CDCl_3$, 300 MHz, ppm), δ = 1.80-1.94 (m, 4 H; $(CH_2)CH_2(CH_2)$), 2.42-2.64 (m, 4 H; $ArCH_2(CH_2)$), 3.37 (t, *J* = 5.5, 4 H; $NCH_2(CH_2)$), 3.70-3.98 (m, 7 H; $NCH_2(CF_3)$ and OCH_3), 6.24 (s, 2 H), 6.46 (s, 2 H), 7.79 (br. s, 1 H; H-7'), 8.04 (d, J = 8.0, 1 H; H-4'), 8.25 (dd, J = 1.3 and 8.0, 1 H; H-5'). ESI-MS, positive mode: *m*/*z* (rel. int., %) = 633 (100) $[M+H]^+$, 655 (20) $[M+Na]^+$. $C_{32}H_{26}F_6N_2O_5$, HR-MS (ESI, positive mode): 633.1816 $[M+H]^+$ (found), 633.1819 (calculated).

[0084] Diazaketone 29: $(COCl)_2$ (0.5 mL, 6 mmol) was added to a suspension of compound **28** (0.33 g, 0.5 mmol) in CH_2Cl_2 (5 mL) with stirring at room temperature. The mixture was kept at room temperature for 0.5 h. Then the solvent and excess of $(COCl)_2$ were removed in vacuo. The residue was dissolved in THF (4 mL). Et_3N (0.7 mL, 5 mmol) and a solution of Me_3SiCHN_2 in Et_2O (1 mL of 2 M solution, 2 mmol) were added to the reaction mixture at with stirring and cooling with a water bath. The reaction mixture was stirred at room temperature for 14 h. Then the solvent and excess of Me_3SiCHN_2 were removed in vacuo, and the title product was isolated from the residue by column chromatography (CH_2Cl_2 / MeOH (10:1)). Additional purification by column chromatography with another eluent (CH_2Cl_2 / *n*-hexane (2:1)) afforded the pure compound **29** (25 mg, 8%). HPLC: t_R = 22.5 min. ^{1}H NMR ($CDCl_3$, 300 MHz, ppm), δ = 1.76-1.92 (m, 4H; $(CH_2)CH_2(CH_2)$), 2.42-2.64 (m, 4 H; $ArCH_2(CH_2)$), 3.26-3.40 (m, 4 H; $NCH_2(CH_2)$), 3.70-3.97 (m, 7 H; $NCH_2(CF_3)$ and OCH_3), 6.36 (s, 2 H), 6.40 (s, 2 H), 7.67 (br. s, 1 H; H-7'), 7.87 (d, *J* = 8.0, 1 H; H-4'), 8.07 (dd, J = 1.4 and 8.0, 1 H; H-5'). ^{13}C NMR ($CDCl_3$, 75.5 MHz, ppm), δ = 21.7 $(CH_2)CH_2(CH_2)$), 27.2 $(ArCH_2(CH_2))$, 48.9 (C; C-1'), 50.8 $(NCH_2(CH_2))$, 52.4 (OCH_3), 53.6 (q, *J* = 33; $NCH_2(CF_3)$), 79.0 (CN_2), 98.7 (CH; C-4/5), 108.6 (C), 119.4 (C), 122.2 (CH), 125.5 (q, *J* = 281; CF_3), 126.9 (CH), 128.0 (CH; C-1/8), 129.7 (CH), 135.7 (C), 138.2 (C), 145.2 (C), 150.4 (C), 155.9 (C), 166.1 (CO_2CH_3), 186.2 (CO). ESI-MS, positive mode: *m*/*z* (rel. int., %) = 657 (17) $[M+H]^+$, 679 (100) $[M+Na]^+$. $C_{33}H_{26}F_6N_4O_4$, HR-MS (ESI, positive mode): 657.1930 $[M+H]^+$ (found), 657.1931 (calculated).

[0085] Carboxylic acid 30: Ester **29** (25 mg, 0.039 mmol) was dissolved in THF (4 mL); H_2O (1 mL) and MeOH (5 mL) were added under N_2. Then 1 M aq. NaOH (0.5 mL) was added to the reaction mixture and it was kept at room temperature for 16 h. Then the reaction mixture was acidified with 1 M aq. HCl (0.5 mL), volatile solvents were removed in vacuo, and the residue was dissolved in H_2O (20 mL). The solution was carefully acidified with 1 M aq. HCl to pH = 6, and the title product was extracted with CH_2Cl_2. The organic solutions were dried with Na_2SO_4, and the solvent was removed in vacuo. Yield - 24 mg (ca. 100%). HPLC: t_R = 18.0 min. ^{1}H NMR ($CDCl_3$, 300 MHz, ppm), δ = 1.76-1.92 (m, 4H; $(CH_2)CH_2(CH_2)$), 2.42-2.64 (m, 4 H; $ArCH_2(CH_2)$), 3.26-3.40 (m, 4 H; $NCH_2(CH_2)$), 3.81 (q, *J* = 9. 0, 4 H; $NCH_2(CF_3)$), 6. 36 (s, 2 H), 6. 39 (s, 2 H), 7.71 (br. s, 1 H; H-7'), 7.89 (d, *J* = 7.2, 1 H; H-4'), 8.09 (br. d, *J* = 7.2, 1 H; H-5'). ^{13}C NMR ($CDCl_3$, 125.7 MHz, ppm), δ = 21.8 $(CH_2)CH_2(CH_2)$), 27.2 $(ArCH_2(CH_2))$, 49.0 (C; C-1'), 50.8 $(NCH_2(CH_2))$, 53.6 (q, *J* = 33; $NCH_2(CF_3)$), 78.4 (CN_2), 98.8 (CH; C-4/5), 108.5 (C), 119.4 (C), 122.3 (CH), 125.5 (q, *J* = 284; CF_3), 127.5 (CH), 127.9 (CH; C-1/8), 130.2 (CH), 134.9 (C), 138.7 (C), 145.3 (C), 150.5 (C), 156.0 (C), 170.0 (CO_2H), 186.2 (CO). ESI-MS, positive mode: *m*/*z* (rel. int., %) = 665 (100) $[M+Na]^+$, 1307 (50) $[2M+Na]^+$. $C_{32}H_{24}F_6N_4O_4$, HR-MS (ESI, positive mode): 665.1592 $[M+Na]^+$ (found), 665.1594 (calculated).

[0086] 1,2,3-Thiadiazole 31: A mixture of compound **10** (53 mg, 0.11 mmol), Lawesson's reagent (48 mg, 0.12 mmol), and benzene (3 mL) was refluxed under N_2 for 1 h. The reaction mixture was cooled, and the solution was decanted from the tarry residue. The solvent was evaporated under reduced pressure, and the crude product was isolated from the residue by column chromatography ($CHCl_3$/*n*-hexane, 1:1), yield - 13 mg (24%). Another portion of the title product **31** was isolated from the tarry residue by column chromatography ($CHCl_3$/MeOH 20:1); total yield - 27 mg (50%). HPLC (A/B: 70/30 $\rightarrow$ 0/100 in 25 min): t_R = 8.1 min.

^{1}H NMR ($CDCl_3$, 300 MHz, ppm), δ = 1.12 (t, *J* = 7.1, 12 H; $(CH_2)CH_3$), 3.29 (q, *J* = 7.1, 8 H; $NCH_2(CH_3)$), 6.06-6.24 (m, 4 H), 6.47 (br.s, 2 H), 7.27-7.40 (m, 3 H), 7.42-7.48 (m, 1 H), 7.66 (br. d, *J* = 6.8, 1 H). ESI-MS, positive mode: *m*/*z* (rel. int., %) = 483 (90) $[M+H]^+$, 505 (5) $[M+Na]^+$, 965 (100) $[2M+H]^+$. $C_{29}H_{30}N_9OS$, HR-MS (ESI, positive mode): 505.2036 $[M+Na]^+$ (found), 505.2033 (calculated).

[0087] Photolysis of 1,2,3-thiadiazole 31: A solution of compound **31** (60 mg, 0.12 mmol) in MeOH (200 mL) was placed into a photolysis reactor, and nitrogen was bubbled through a solution for 30 min at room temperature with stirring. Then the reaction mixture was irradiated at ambient temperature with stirring under N_2 for 2.5 h, until the starting compound could not be detected by HPLC. The solvent was removed in vacuo. More then 10 new compounds were detected in the reaction mixture (HPLC). The main product - thioester **32 -** was isolated by preparative HPLC as a dark red solid. HPLC (A/B: 70/30 $\rightarrow$ 0/100 in 25 min): t_R = 20.6 min. UV (MeOH): λ_{max} = 559 nm, λ_{em} = 580 nm, Φ_{fl} = 0.46. ESI-MS, positive mode: *m*/*z* (rel. int., %) = 487 (100) $[M]^+$. $C_{30}H_{35}N_2O_2S$ (+), HR-MS (ESI, positive mode): 487.2405 $[M]^+$ (found), 487.2414 (calculated).

EXAMPLE 2

*Imaging subcellular structures with the new caged fluorescent dye compounds **22**/**23***

[0088] For imaging subcellular structures with photoactivable dyes in living cells, the photoactivable dyes have to fulfil (at least) two criteria: i) the fluorescent dyes must be able to pass cellular membranes and ii) the photoactivation process

must be possible under aqueous conditions (i.e. cell culture medium or other live compatible buffered solutions). To determine if compounds **22/23** meets these criteria, mammalian PtK2 cells were incubated for 2 hours in ~1 ml of staining solution (1 ml culture medium containing 3 μl of the saturated solution of compounds **22/23**-NHS in DMSO) under cell culture conditions and subsequently for 5 minutes in cell culture medium without dye (for washing). After this, the stained cells were imaged before and after photoactivation (Fig. 1A). Imaging (exc.: 570/20 nm; det.: 640/40 nm; intensity: 30 μW/ imaged area; exposure time: 20 ms) and switching (activation: 420/30 nm; intensity in the sample: 8 μW/ imaged area; exposure time: 20 ms) was performed in an epifluorescence microscope (DM6000B; equipped with a 100 x 1.4 NA oil objective and a mercury arc lamp; Leica Microsystems, Wetzlar, Germany). Thereby we found that compounds **22/23** penetrate the cell and stain predominantly mitochondria in living cells. It is readily photoactivatable. Thus, compounds **22/23** meet both criteria for live cell imaging named above.

[0089] Next, the switching behaviour of compounds **22/23** in apolar, buffered mounting media like Mowiol (with DABCO) was determined. These embedding media are frequently utilized for imaging fixed cells which are labelled by immunocytochemistry or related approaches. To check the new compound for photoactivation in Mowiol, the microtubule cytoskeleton of formaldehyde fixed cells was immunolabeled according to standard procedures. After embedding in Mowiol (containing DABCO), the samples were imaged and switched as described above. It was found that compounds **22/23**-NHS can readily be coupled to antibodies that retain their ability to be used for immunocytochemistry. Furthermore, it was found that compounds **22/23** may be photoswitched in Mowiol enabeling imaging in fixed and embedded cells (Fig. 1B).

[0090] Analyzing the images, the present inventors determined the increase of the fluorescence signal upon switching to be of a factor of > 40 in living cells. In fixed cells embedded in Mowiol, a relatively larger fraction of non-irradiated compounds **22/23** was in the fluorescent state. The increase of the fluorescence signal upon switching was around 10 fold.

[0091] Fig. 1. illustrates the switching of compounds **22/23** in living and fixed eukaryotic cells. (A) Compounds **22/23** switched in living cells. For staining, mammalian Ptk2 cells were incubated for 2 hours in ~1 ml of staining solution (1 ml culture medium containing 3 μl of saturated solution of 22/23-NHS in DMSO) under cell culture conditions. Under these conditions the mitochondria are preferentially labelled. Imaging (exc.: 570/20 nm; det.: 640/40 nm; intensity: 30 μW/ imaged area; exposure time: 20 ms) and switching (activation: 420/30 nm; intensity: 8 μW/ imaged area; exposure time: 20 ms) were performed with an epifluorescence microscope (equipped with a mercury arc lamp). After switching of living cells, the fluorescence signal rises by a factor of > 40. (B) Compounds **22/23**-NHS were be coupled to antibodies, introduced into fixed cells and switched in Mowiol. Here, the microtubule cytoskeleton was labelled. After embedding in Mowiol containing DABCO, the samples were imaged and switched as indicated above. After switching the fluorescence signal rises by a factor of ~ 10. For comparison, the color table was kept constant for the respective images.

EXAMPLE 3

Determination of intensity-dependent switching kinetics

[0092] To determine the photoinduced switching kinetics of compounds **22/23,** the inventors immunolabelled the tubulin cytoskeleton in fixed mammalian cells with secondary antibodies coupled with compounds **22/23**-NHS (as indicated above), and mounted the cells in Mowiol containing DABCO. Then the switching kinetics were determined while photoactivating the dye either with low light intensities (3 μW/ imaged area) or higher light intensities (70 μW/ imaged area). To this end the samples were subjected to several imaging-switching cycles. For the low light intensities each cycle consisted of 200 ms of imaging (exc.: 570/20 nm; det.:640/40 nm; intensity: 11 μW/ imaged area) and 1 ms of switching (activation: 420/30 nm; intensity: 3 μW/ imaged area). For the higher light intensities imaging and switching was performed as indicated above, but exposure times and light intensities were modified (Imaging: exc.: 570/20 nm; det.: 640/40 nm; intensity: 200 μW/ imaged area; exposure time: 20 ms; Activation 420/30 nm; intensity: 70 μW/ imaged area; exposure time: 1 ms). The fluorescence signal of the individual images was summed up and normalized to the brightest image and displayed in a graph (Fig. 2). Using the lower light intensity, the course of the increase in the fluorescence signal follows saturation kinetics. In case of the higher light intensities, after 15 ms switching the fluorescence signal decreases after an initial raise, likely because of photobleaching superseding photoactivation. The kinetic data demonstrate that compound(s) **22/23** may be utilized for various applications including superresolution microscopy and protein tracking applications.

[0093] Fig. 2 demonstrates intensity dependent switching kinetics. (A) Kinetics under low light intensities. Immunolabeling of the microtubule cytoskeleton of formaldehyde fixed cells with the secondary antibodies coupled with compounds **22/23**-NHS was performed according to standard procedures. To evaluate the switching kinetics, after embedding in Mowiol (containing DABCO), the samples were subjected to several imaging-switching cycles using an epifluorescence microscope (DM6000B, equipped with a 100 x 1.4 NA oil objective and a mercury arc lamp; Leica Microsystems, Wetzlar, Germany). Each cycle consisted of 200 ms of imaging (exc.: 570/20 nm; det.:640/40 nm; intensity: 11 μW/ imaged area) and 1 ms of switching (activation.: 420/30 nm; intensity: 3 μW/ imaged area). The brightness of the individual images

was summed up and normalized to the brightest image and displayed in the corresponding graph. (B) Kinetics under higher light intensities. Staining, imaging and switching was performed as described above, but exposure times and light intensities were modified (Imaging: exc.: 570/20 nm; det.:640/40 nm; intensity in sample: 200 μW/ imaged area; exposure time: 20 ms; activation: 420/30 nm; intensity in sample: 70 μW/ imaged area; exposure time: 1 ms). Analysis of the kinetics of photoactivation as in (A). For comparison, the color table was kept constant.

EXAMPLE 4

Tracking of proteins and organelles within living cells

[0094] To determine whether the dynamics of proteins, organelles and other subcellular structures can be analyzed with compound **22/23,** living mammalian PtK2 cells were stained with compound **22/23**-NHS as described above. Switching and imaging was performed using a confocal microscope (TCS SP5 equipped with a 1,4 NA oil immersion objective, a 405 nm diode laser and a 461 nm diode laser, respectively, Leica Microsystems, Mannheim, Germany). The whole field of view was imaged, but only a part of the field of view (region of interest, ROI) was irradiated with 405 nm to activate compound **22/23.** The photomultiplier sensitivity was set in a way, that before switching no fluorescence signal was detectable upon irradiation with 561 nm laser light (Fig. 3). After 30 ms switching with 405 nm laser light (within the ROI) brightly fluorescent structures - predominantly mitochondria and vesicles - were visible within photoactivated areas. After 330 ms of further irradiation, bright structures were not only visible within switched areas but also in the rest of the cell. The fluorescent structures outside of the ROI are due to movement of the highlighted structures within the cells, demonstrating that compound **22/23** can be used for live cell tracking experiments.

[0095] Fig.3 illustrates the tracking of proteins and organelles within living cells. Prior to imaging and switching in a confocal microscope (TCS SP5, Leica Microsystems, Mannheim, Germany), mammalian PtK2 cells were incubated for 2 hours in ~1 ml of staining solution (1 ml culture medium containing 3 μL of the saturated solution of **22/23**-NHS in DMSO) under cell culture conditions. (A) Before switching (t = 0) no fluorescence signal was detected upon irradiation with 561 nm laser light. After 30 ms switching (t = 30'') with 405 nm laser light within the ROI (indicated by a dashed box), a strong fluorescence signal was visible within the photoactivated areas. After 330 ms of further irradiation (t = 360''), bright structures were not only visible within switched areas but also in the rest of the cell. (B) Close-up of (A) t = 363" to t = 390". Movement of individual vesicles, containing photactivated compound **22/23** is visible and can be tracked within living cells. For comparison, the color table was kept constant.

EXAMPLE 5

Single molecule switching experiments

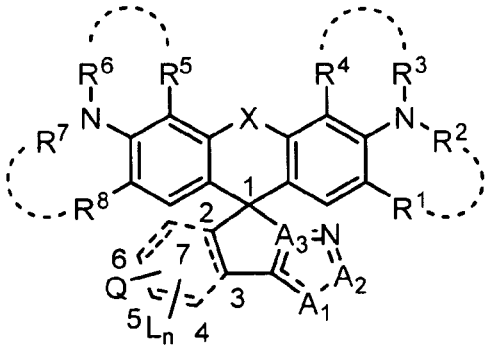
[0096] To successfully use a dye in single molecule switching (SMS) experiments, it is essential that the dye can be switched between a dark and a bright state, and that one of these transitions is light-driven. Furthermore, to achieve high localisation accuracy, it is necessary that a single molecule emits a large number (thousands) of photons before being bleached or being switched off. The localisation accuracy (and thus the resolution enhancement) is inversely proportional to the square root of the number of emitted photons.

[0097] Both of these prerequisites for SMS are satisfactorily fulfilled by the compound **22/23.** To demonstrate this, the microtubule cytoskeleton in fixed mammalian cells was immunolabelled with the secondary antibodies bearing the residues of the compound **22/23** (as described above), and the cells were coated with PVA. The samples were imaged in a widefield setup with a fast EMCCD-camera using a 532 nm laser for excitation and a 375 nm laser for "uncaging". Thereby we could apply compound **22/23** successfully for imaging of the microtubule cytoskeleton with SMS. The measured localisation accuracy in the lateral directions was at least ~55 nm (~ 4 times better than the resolution of confocal microscopes and more than 5 times better than the diffraction limited resolution of the used microscope).

[0098] Figure 4. Conventional microscopic image (A) and single molecule switching (SMS) nanoscopic image (B) of the microtubule network in a whole fixed PtK2 cell immunolabelled with secondary antibodies conjugated with compounds **22/23-**NHS. For preparation of the samples, mammalian PtK2 cells were grown on cover slips, fixed with formaldehyde, extracted, blocked and finally immunolabelled using a tubulin specific primary plus a dye labelled secondary antibody. After coating with PVA, the samples were imaged using a 532 laser for excitation and a 375 nm laser for switching. The left side of the cell (A) is displayed as superposition (an "add up") of the intensity of 28000 frames taken in this experiment, exemplifying the resolution, a conventional diffraction-limited image would achieve. The right side of the same cell (B) is displayed as histogram representation of the acquired data providing the diffraction-unlimited optical super-resolution. Using compound **22/23** in SMS, a localisation accuracy of at least ~55 nm was measured by analysing the data and evaluating an agglomeration of dye molecules.

Claims

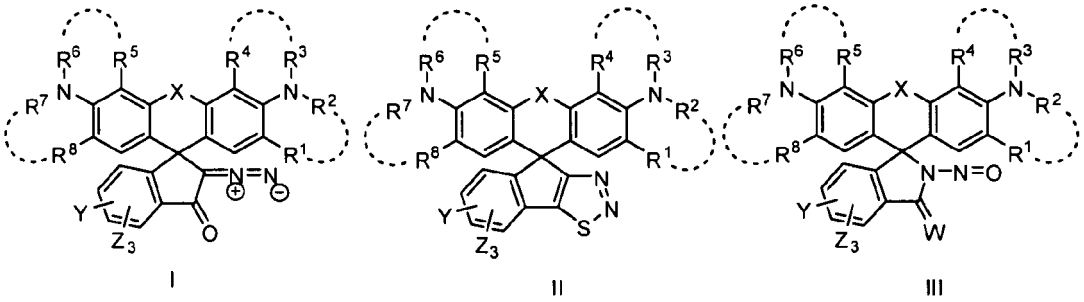
1. Photoactivable rhodamine or carbopyronine derivatives of the following general formulae **G1-G4** comprising UV light absorbing chromophores which in the course of photolysis by irradiation at 254-490 nm eliminate nitrogen or nitrogen(I) oxide and generate fluorescent rhodamine or carbopyronine dyes



G1 - G4

G1: A1 = 0, A2 = N, A3 = C;
G2: A1 = S, A2 = N, A3 = C;
G3: A1 = O, A2 = O, A3 = N;
G4: A1 = S, A2 = O, A3 = N;
wherein R^1, R^2, (R^1-R^2), R^3, R^4, (R^3-R^4), R^5, R^6, (R^5-R^6), R^7, R^8, (R^7-R^8) = H, unsubstituted or substituted alkyl (cycloalkyl), unsubstituted or substituted cycloalkenyl, unsubstituted or substituted heterocylic residue, unsubstituted or substituted aryl, unsubstituted or substituted hetaryl; and X = O, S, $C(CH_3)_2$;
the chemical bond between C-2 and C-3 may be a single or *cis*-double bond;
alternatively, C-2 and C-3 may be a part of any cyclic system, preferably the benzene ring;
and the substituents Q and/or L are independently defined as follows (n = 0, 1, 2, 3 or 4): H, $COOR^9$, COCH=N=N, CH_2COOR^9, $N(R^1...R^2)$, $NR^{10}R^{11}$, SR^{12}, F, Cl;
wherein R^9 = H, unsubstituted or substituted alkyl, cycloalkyl, or heterocyclic residue, *N*-succinimidyl, N-sulfosuccinimidyl, unsubstituted or substituted phenyl, aryl or hetaryl; R^{10}, R^{11} = C=S, C=O; R^{12} = H, $(CH_2)_mCOOR^9$ (*m* = 1 - 11), unsubstituted or substituted alkyl, cycloalkyl, heterocyclic residue, aryl or hetaryl.

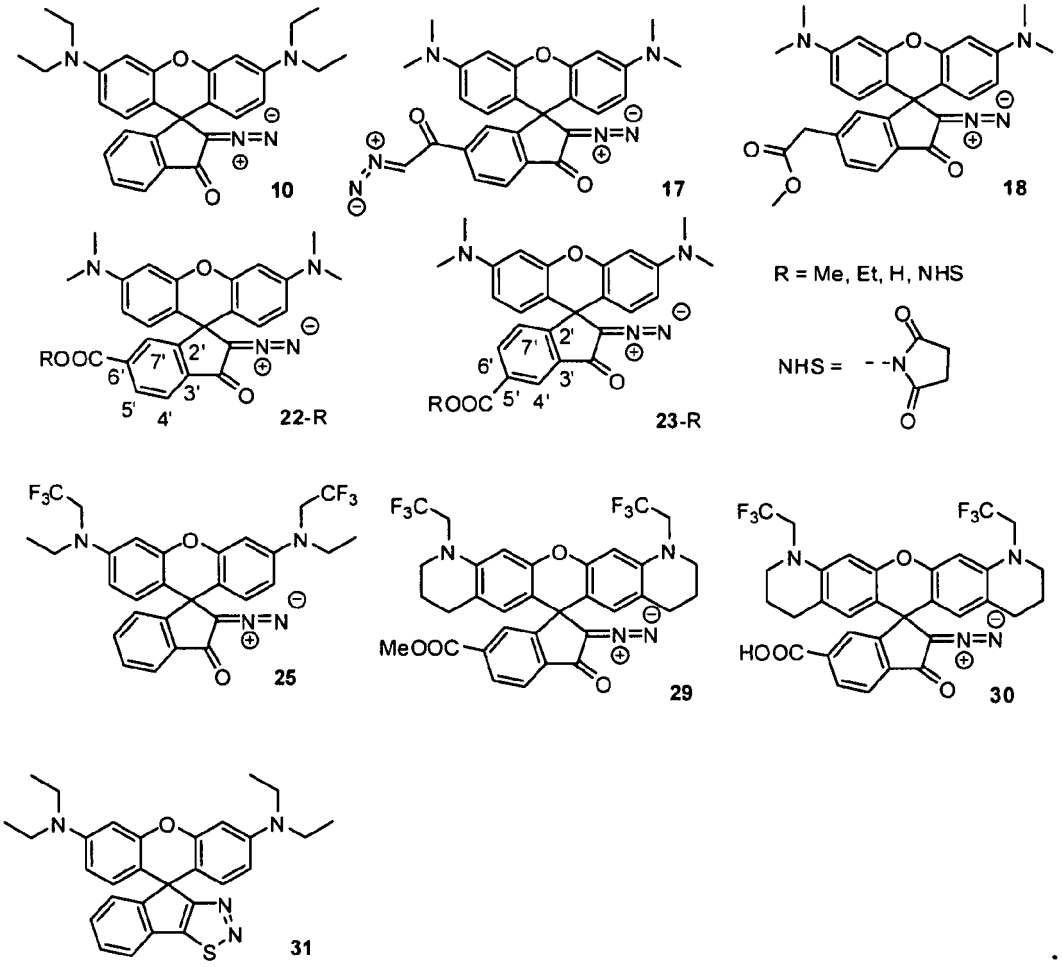
2. Compounds according to claim 1 of the general formulae **I-III**



I II III

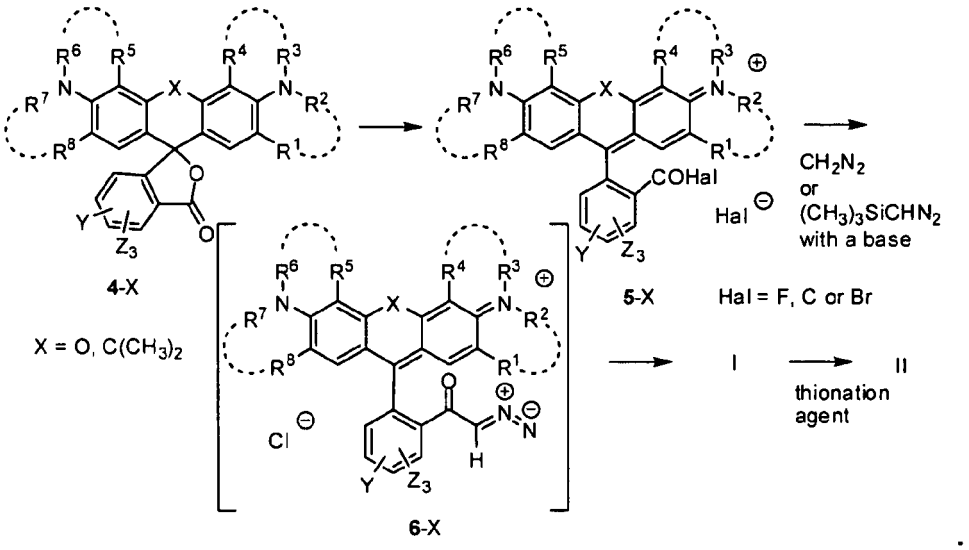
wherein R^1, R^2, (R^1-R^2), R^3, R^4, (R^3-R^4), R^5, R^6, (R^5-R^6), R^7, R^8, (R^7-R^8) = H, unsubstituted or substituted alkyl (cycloalkyl), unsubstituted or substituted cycloalkenyl, unsubstituted or substituted heterocylic residue, unsubstituted or substituted aryl, unsubstituted or substituted hetaryl; and X = O, S, $C(CH_3)_2$; Y = $COOR^9$, COCH=N=N, CH_2COOR^9, $N(R^1...R^2)$, $NR^{10}R^{11}$, SR^{12}, F, Cl; Z = H, F, Cl; W = O, S; R^9 = H, unsubstituted or substituted alkyl, cycloalkyl, or heterocyclic residue, N-succinimidyl, N-sulfosuccinimidyl, unsubstituted or substituted phenyl, aryl or hetaryl; R^{10}, R^{11} = C=S, C=O; R^{12} = H, $(CH_2)_mCOOR^9$ (*m* = 1 - 11), unsubstituted or substituted alkyl, cycloalkyl, heterocyclic residue, aryl or hetaryl.

3. Compounds according to claim 2 selected from the group of compounds 10, 17, 18, 22-Me, 23-Me, 22-Et, 23-Et, 22-H, 23-H, 22-NHS, 23-NHS, 25, 29, 30 and 31 below



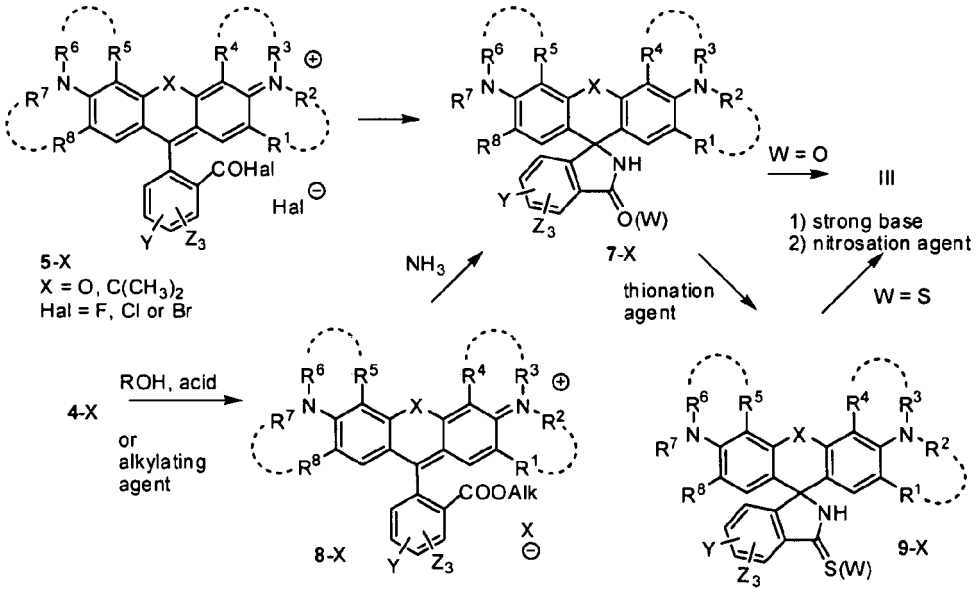
4. A method for preparing compounds **I** and **II** according to claim 2, comprising the steps:

a) converting the spiro-9*H*-xanthene or 9,10-dihydroanthracene starting compound **4-X** as depicted in the following scheme (or their synthetically equivalent open forms having a free carboxylic group) into the corresponding acid halide **5-X;**
b) reacting compound **5-X** with a diazomethane compound to produce the diazoketone **I**;
c) optionally reacting compound **I** with a thionating agent to produce the 1,2,3-thiadiazole derivative **II**



5. A method for preparing compounds **III** according to claim 2, comprising the steps:

a) converting the acid halide **5-X** or alkyl ester **8-X** as depicted in the following scheme into the amide **7-X;**
b) reacting compound **7-X** with a thionation agent to produce the corresponding thioamide **9-X;**
c) deprotonation of the amide **7-X** or thioamide **9-X** with a strong base followed by the reaction with a nitrosation agent



6. Use of the compounds according to claims 1-3 as reagents for conjugation or bioconjugation.

7. The use according to claim 6 wherein the conjugation or bioconjugation comprises formation of at least one covalent chemical bond or at least one molecular complex with a chemical entity or substance, such as amine, carboxylic acid, aldehyde, alcohol, aromatic compound, heterocycle, dye, amino acid, amino acid residue coupled to any chemical entity, peptide, protein, carbohydrate, nucleic acid, toxin, lipid.

8. The use according to claim 7 wherein the conjugation or bioconjugation comprises formation of two or more covalent chemical bonds and/or molecular complexes.

9. The use according to claim 8 wherein compounds **22**-NHS, **23**-NHS, **17** and **30** with two functional groups are used

as (cross)linking reagents.

10. Use of compounds according to any one of claims 1-3 or of their conjugates for bioconjugation with antibodies and immunolabelling.

11. Use of compounds according to any one of claims 1-3 or of their conjugates as photoactivable fluorescent dyes.

12. Use of the compounds according to any one of claims 1-3 or of their conjugates as cell permeable substances penetrating through membranes of living and fixed cells in the masked non-fluorescent form represented by the structural formulae **G1 - G4.**

13. Use of the compounds according to any one of claims 1-3 or of their conjugates as such or after photoactivation for tracking and monitoring dynamic processes in a sample or in an object.

14. The use according to claim 13 wherein changes in the shape, dimensions and/or the intensity of the fluorescence signal obtained after photoactivation of the compounds according to any one of claims 1-3 or of their conjugates correspond to changes of the sample or object or of its environment.

15. Use of the compounds according to any one of claims 1-3 or of their conjugates as such or after photoactivation as labels in optical microscopy and imaging techniques, microfluidic devices, capillary electrophoresis, protein tracking techniques.

16. The use according to claim 15 wherein the optical microscopy and imaging techniques comprise stimulated emission depletion microscopy [STED], single molecule switching (SMS) "nanoscopy" (diffraction unlimited optical resolution by using switching of the fluorescence of the single molecules, such as single molecule localization microscopy [SMLM], photoactivation localization microscopy [PALM, PALMIRA, fPALM], stochastic optical reconstruction microscopy [STORM]), fluorescence correlation spectroscopy [FCS], fluorescence recovery after photobleaching [FRAP], fluorescence lifetime imaging [FLIM], ground state depletion with individual molecular return [GSDIM], and fluorescence resonant energy transfer [FRET].

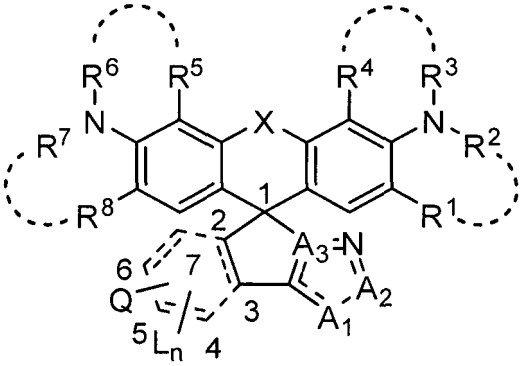
17. Use of the compounds of the compounds according to any one of claims 1-3 or of their conjugates as such or after photoactivation as labels specific for mitochondria.

18. The use according to claim 17 wherein compounds **22-**H, **22**-NHS, **23**-H, and **23**-NHS are used as labels specific for mitochondria.

19. Use of compounds **22**-H, **22**-NHS, **23**-H and **23**-NHS in single molecule localization microscopy.

Patentansprüche

1. Photoaktivierbare Rhodamin- oder Carbopyronin-Derivate der folgenden allgemeinen Formeln **G1-G4,** welche UV-Licht absorbierende Chromophore umfassen, die im Verlauf einer Photolyse durch Bestrahlung bei 254-490 nm Stickstoff oder Stickstoff(I)-Oxid eliminieren und fluoreszierende Rhodamin- oder Carbopyronin-Farbstoffe bilden

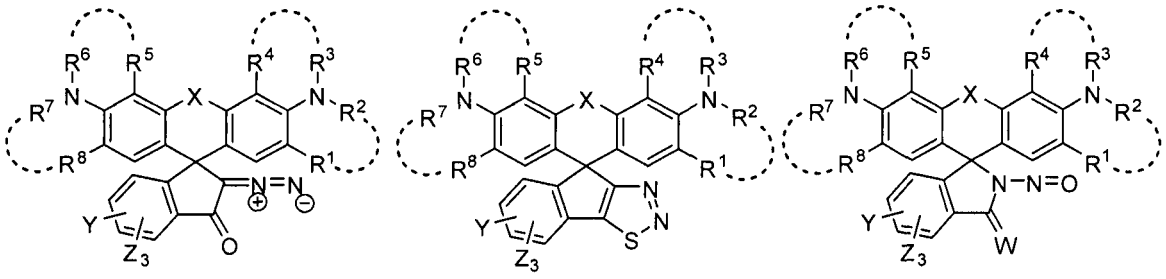


G1 - G4

G1: A1 = O, A2 = N, A3 = C;
G2: A1 = S, A2 = N, A3 = C;
G3: A1 = O, A2 = O, A3 = N;
G4: A1 = S, A2 = O, A3 = N;

wobei R^1, R^2, (R^1-R^2), R^3, R^4, (R^3-R^4), R^5, R^6, (R^5-R^6), R^7, R^8, (R^7-R^8) = H, unsubstituiertes oder substituiertes Alkyl (Cycloalkyl), unsubstituiertes oder substituiertes Cycloalkenyl, ein unsubstituierter oder substituierter heterocyclischer Rest, unsubstituiertes oder substituiertes Aryl, unsubstituiertes oder substituiertes Hetaryl; und X = 0, S, $C(CH_3)_2$;
die chemische Bindung zwischen C-2 und C-3 eine Einfachbindung oder cis-Doppelbindung sein kann;
alternativ C-2 und C-3 Teil eines beliebigen cyclischen Systems, vorzugsweise des Benzolrings, sein können;
und die Substituenten Q und/oder L unabhängig definiert sind wie folgt (n = 0, 1, 2, 3 oder 4): H, $COOR^9$, COCH=N=N, CH_2COOR^9, $N(R^1...R^2)$, $NR^{10}R^{11}$, SR^{12}, F, Cl;
wobei R^9 = H, unsubstituiertes oder substituiertes Alkyl, Cycloalkyl oder ein unsubstituierter oder substituierter heterocyclischer Rest, N-Succinimidyl, N-Sulfosuccinimidyl, unsubstituiertes oder substituiertes Phenyl, Aryl oder Hetaryl; R^{10}, R^{11} = C=S, C=O; R^{12} = H, $(CH_2)_mCOOR^9$ (m = 1 - 11), unsubstituiertes oder substituiertes Alkyl, Cycloalkyl, Aryl oder Hetaryl oder ein unsubstituierter oder substituierter heterocyclischer Rest.

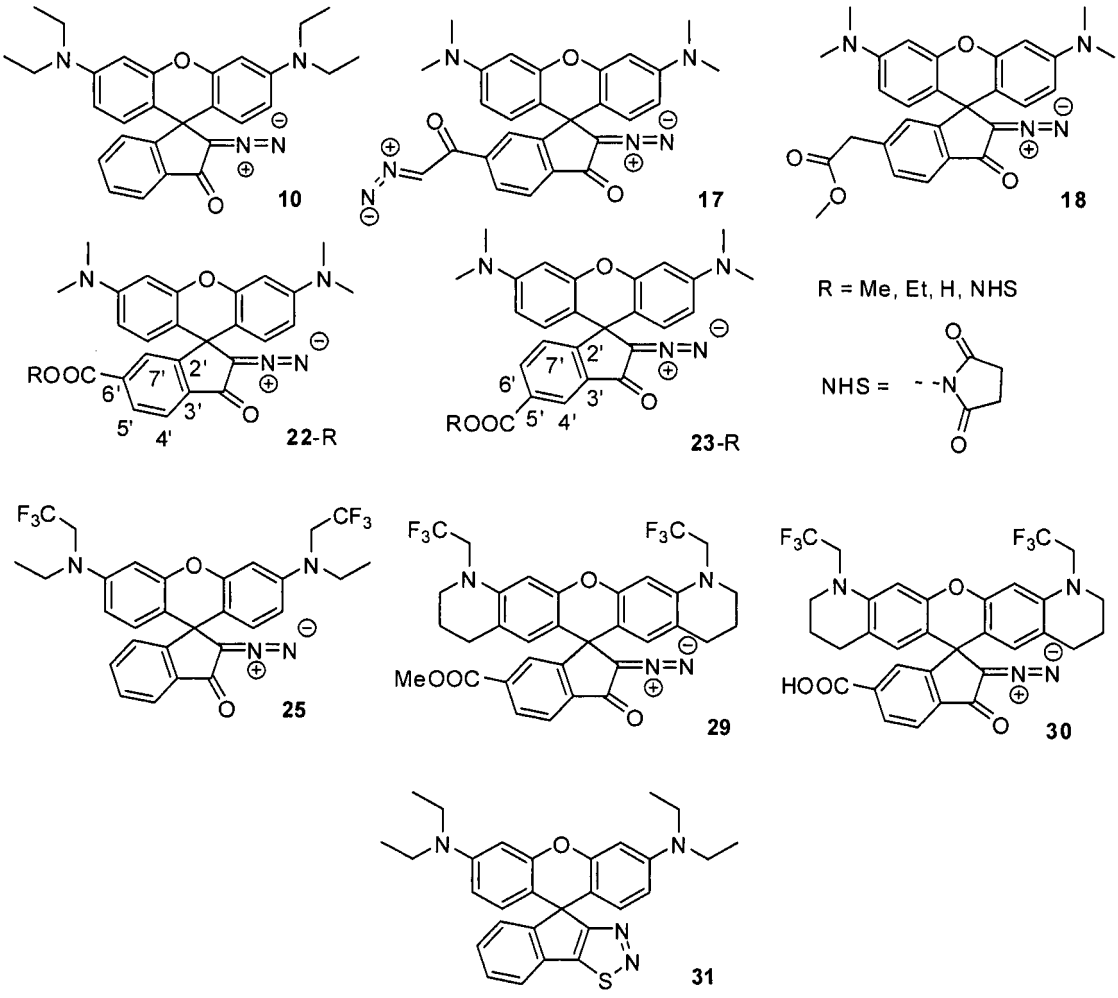
2. Verbindungen nach Anspruch 1 der allgemeinen Formeln **I-III**



I II III

wobei R^1, R^2, (R^1-R^2), R^3, R^4, (R^3-R^4), R^5, R^6, (R^5-R^6), R^7, R^8, (R^7-R^8) = H, unsubstituiertes oder substituiertes Alkyl (Cycloalkyl), unsubstituiertes oder substituiertes Cycloalkenyl, ein unsubstituierter oder substituierter heterocyclischer Rest, unsubstituiertes oder substituiertes Aryl, unsubstituiertes oder substituiertes Hetaryl; und X = O, S, $C(CH_3)_2$; Y = $COOR^9$, COCH=N=N, CH_2COOR^9, $N(R^1...R^2)$, $NR^{10}R^{11}$, SR^{12}, F, Cl; Z = H, F, Cl; W = O, S; R^9 = H, unsubstituiertes oder substituiertes Alkyl, Cycloalkyl, oder ein unsubstituierter oder substituierter heterocyclischer Rest, N-Succinimidyl, N-Sulfosuccinimidyl, unsubstituiertes oder substituiertes Phenyl, Aryl oder Hetaryl; R^{10}, R^{11} = C=S, C=O;
R^{12} = H, $(CH_2)_mCOOR^9$ (m = 1 - 11), unsubstituiertes or substituiertes Alkyl, Cycloalkyl, Aryl or Hetaryl oder ein unsubstituierter oder substituierter heterocyclischer Rest.

3. Verbindungen nach Anspruch 2, ausgewählt aus der Gruppe der folgenden Verbindungen **10, 17, 18, 22**-Me, **23**-Me, **22**-Et, **23**-Et, **22**-H, **23**-H, **22**-NHS, **23**-NHS, **25, 29, 30** und **31**

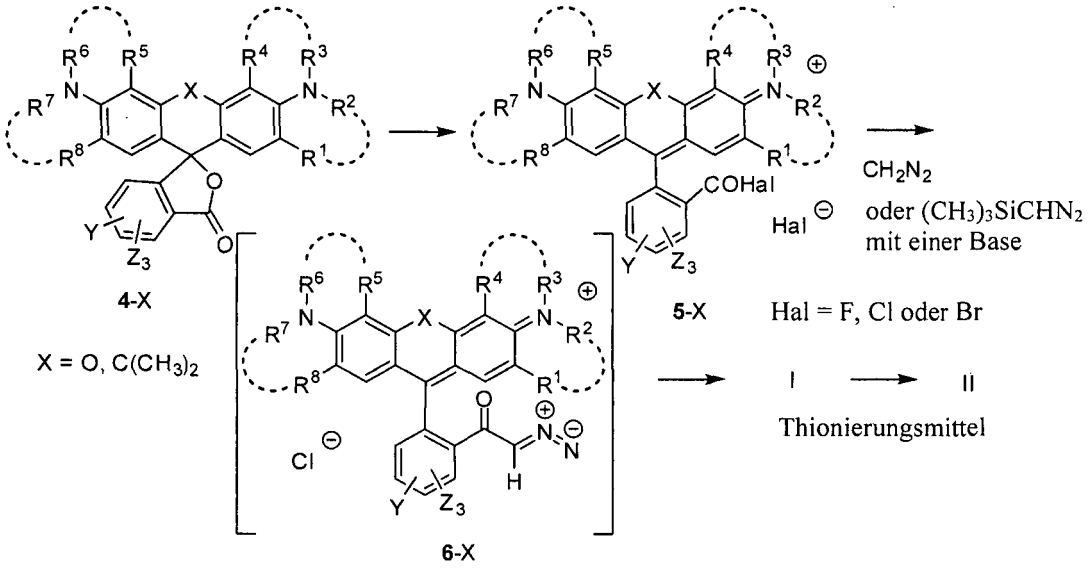


4. Verfahren zur Herstellung von Verbindungen **I** und **II** nach Anspruch 2, umfassend die Schritte:

a) Überführen der Spiro-9*H*-Xanthen- oder 9,10-Dihydro-anthracen-Ausgangsverbindung **4-X** wie im folgenden Schema dargestellt (oder deren synthetisch äquivalenten offenen Formen mit einer freien Carboxylgruppe) in das entsprechende Säurehalogenid **5-X;**

b) Umsetzen der Verbindung **5-X** mit einer Diazomethan-Verbindung, um das Diazoketon **I** zu bilden;

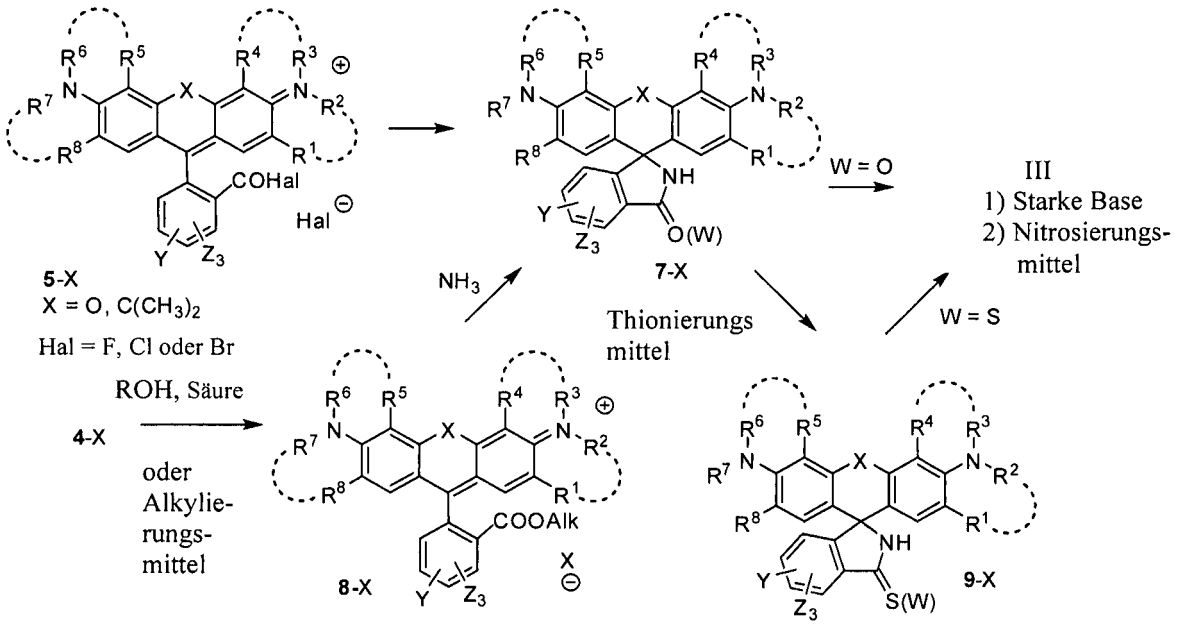
c) gegebenenfalls Umsetzen der Verbindung **I** mit einem Thionierungsmittel, um das 1,2,3-Thiadiazolderivat **II**



zu bilden.

5. Verfahren zur Herstellung von Verbindungen **III** nach Anspruch 2, umfassend die Schritte:

a) Überführen des Säurehalogenids **5-X** oder Alkylesters **8-X** wie im folgenden Schema dargestellt in das Amid **7-X;**
b) Umsetzen der Verbindung **7-X** mit einem Thionierungsmittel, um das entsprechende Thioamid **9-X** zu bilden;
c) Deprotonieren des Amids **7-X** oder Tioamids **9-X** mit einer starken Base, gefolgt von der Reaktion mit einem Nitrosierungsmittel



6. Verwendung der Verbindungen nach den Ansprüchen 1-3 als Reagenzien für die Konjugation oder Biokonjugation.

7. Verwendung nach Anspruch 6, wobei die Konjugation oder Biokonjugation die Bildung von mindestens einer kovalenten chemischen Bindung oder mindestens einem molekularen Komplex mit einer chemischen Gruppierung oder einer Substanz, wie zum Beispiel Amin, Carbonsäure, Aldehyd, Alkohol, aromatische Verbindung, Heterocyclus, Farbstoff, Aminosäure, Aminosäurerest gekoppelt an irgendeine chemische Gruppierung, Peptid, Protein, Kohlenhydrat, Nucleinsäure, Toxin, Lipid, umfasst.

8. Verwendung nach Anspruch 7, wobei die Konjugation oder Biokonjugation die Bildung von zwei oder mehr kovalenten chemischen Bindungen und/oder molekularen Komplexen umfasst.

9. Verwendung nach Anspruch 8, wobei die Verbindungen **22-**NHS, **23**-NHS, **17** und **30** mit zwei funktionalen Gruppen als Vernetzungsreagenzien verwendet werden.

10. Verwendung von Verbindungen nach irgendeinem der Ansprüche 1-3 oder von deren Konjugaten zur Biokonjugation mit Antikörpern und Immunmarkierung.

11. Verwendung von Verbindungen nach irgendeinem der Ansprüche 1-3 oder von deren Konjugaten als photoaktivierbare fluoreszierende Farbstoffe.

12. Verwendung der Verbindungen nach irgendeinem der Ansprüche 1-3 oder von deren Konjugaten als zellpermeable Substanzen, welche in der maskierten, nicht-fluoreszierenden Form, repräsentiert durch die Strukturformeln **G1** - **G4,** die Membranen von lebenden und fixierten Zellen durchdringen.

13. Verwendung der Verbindungen nach irgendeinem der Ansprüche 1-3 oder von deren Konjugaten als solche oder nach Photoaktivierung zur Verfolgung und Überwachung dynamischer Prozesse in einer Probe oder einem Objekt.

14. Verwendung nach Anspruch 13, wobei Veränderungen in der Gestalt, den Dimensionen und/oder der Intensität des nach Photoaktivierung der Verbindungen nach irgendeinem der Ansprüche 1-3 oder deren Konjugate erhaltenen Fluoreszenzsignals mit Veränderungen der Probe oder des Objekts oder deren bzw. dessen Umgebung korrespondieren.

15. Verwendung der Verbindungen nach irgendeinem der Ansprüche 1-3 oder von deren Konjugaten als solche oder nach Photoaktivierung als Marker in Techniken der optischen Mikroskopie und Bildgebung, mikrofluidischen Vorrichtungen, in der Kapillarelektrophorese, in Proteinverfolgungstechniken.

16. Verwendung nach Anspruch 15, wobei die Techniken der optischen Mikroskopie und Bildgebung umfassen stimulierte Emissionsdepletionsmikroskopie [STED], Einzelmolekül-Schaltungs (SMS)-"Nanoskopie" (beugungsunbeschränkte optische Auflösung durch Einsatz einer Schaltung der Fluoreszenz der Einzelmoleküle, wie zum Beispiel Einzelmolekül-Lokalisationsmikroskopie [SMLM], photoaktivierte Lokalisationsmikroskopie [PALM, PALMIRA, fPALM], stochastische optische Rekonstruktionsmikroskopie [STORM]), Fluoreszenzkorrelationsspektroskopie [FCS], Fluoreszenzregneration nach Photobleichung [FRAP], Fluoreszenzlebensdauer-Bildgebung [FLIM], Grundzustandsdepletion mit Einzelmolekülrückkehr [GSDIM] und Fluoreszenz-Resonanzenergietransfer [FRET].

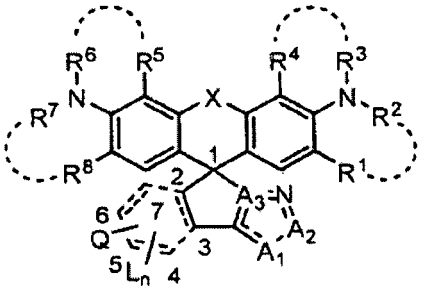
17. Verwendung der Verbindungen nach irgendeinem der Ansprüche 1-3 oder von deren Konjugaten als solche oder nach Photoaktivierung als Mitochondrien-spezifische Marker.

18. Verwendung nach Anspruch 17, wobei die Verbindungen **22**-H, **22**-NHS, **23**-H und **23**-NHS als Mitochondrien-spezifische Marker verwendet werden.

19. Verwendung der Verbindungen **22**-H, **22**-NHS, **23**-H und **23-**NHS bei der Einzelmolekül-Lokalisationsmikroskopie.

Revendications

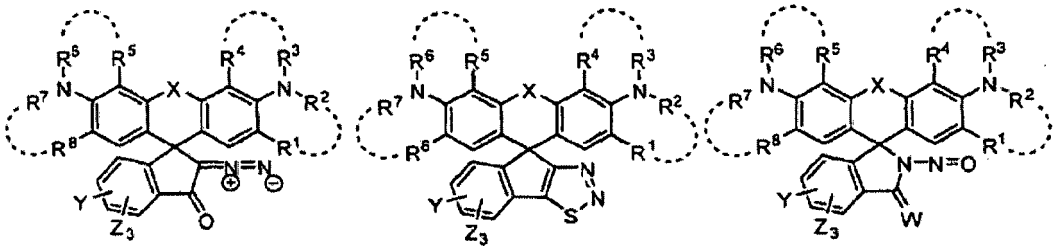
1. Dérivés de rhodamine ou de carbopyronine photo-activables des formules générales G1 à G4 suivantes comprenant des chromophores absorbant la lumière UV qui, au cours de la photolyse par irradiation à 254-490 nm, éliminent de l'azote ou de l'oxyde d'azote (I) et génèrent des colorants de type rhodamine ou carbopyronine fluorescents



G1 - G4

G1 : A1 = O, A2 = N, A3 = C ;
G2 : A1 = S, A2 = N, A3 = C ;
G3 : A1 = O, A2 = O, A3 = N ;
G4 : A1 = S, A2 = O, A3 = N ;
dans lesquelles R^1, R^2, (R^1-R^2), R^3, R^4, (R^3-R^4), R^5, R^6, (R^5-R^6), R^7, R^8, (R^7-R^8) = H, un groupe alkyle (cycloalkyle) non substitué ou substitué, cycloalcényle non substitué ou substitué, un résidu hétérocyclique non substitué ou substitué, aryle non substitué ou substitué, hétéroaryle non substitué ou substitué ; et X = O, S, $C(CH_3)_2$;
la liaison chimique entre C-2 et C-3 peut être une liaison simple ou une double liaison *cis ;*
en variante, C-2 et C-3 peuvent faire partie d'un quelconque système cyclique, de préférence un cycle benzène ;
et les substituants Q et/ou L sont définis indépendamment de la manière suivante (*n* = 0, 1, 2, 3 ou 4) : H, $COOR^9$, COCH=N=N, CH_2COOR^9, $N(R^1...R^2)$, $NR^{10}R^{11}$, SR^{12}, F, Cl ;
dans lesquelles R^9 = H, un groupe alkyle non substitué ou substitué, cycloalkyle, ou un résidu hétérocyclique, N-succinimidyle, N-sulfosuccinimidyle, phényle non substitué ou substitué, aryle ou hétéroaryle ; R^{10}, R^{11} = C=S, C=O ; R^{12} = H, $(CH_2)_mCOOR^9$ (*m* = 1 à 11), alkyle non substitué ou substitué, cycloalkyle, un résidu hétérocyclique, aryle ou hétéroaryle.

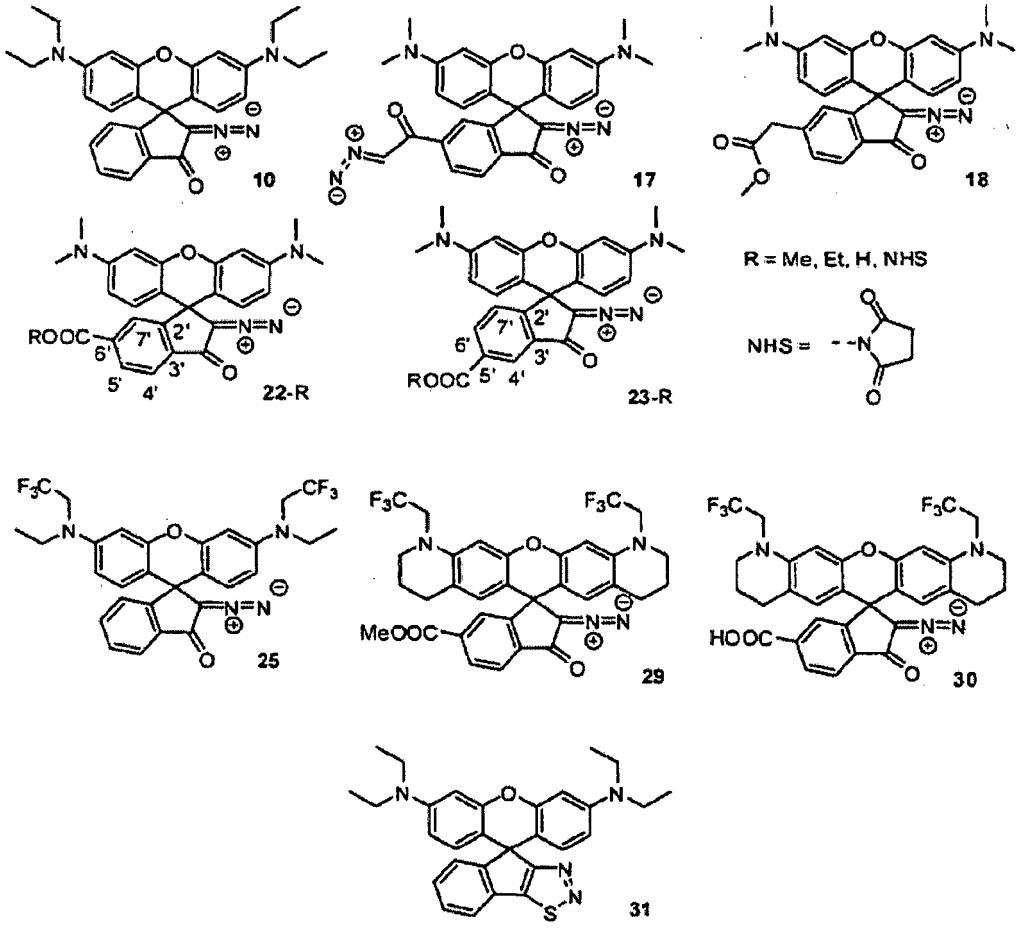
2. Composés selon la revendication 1, des formules générales I à III



I II III

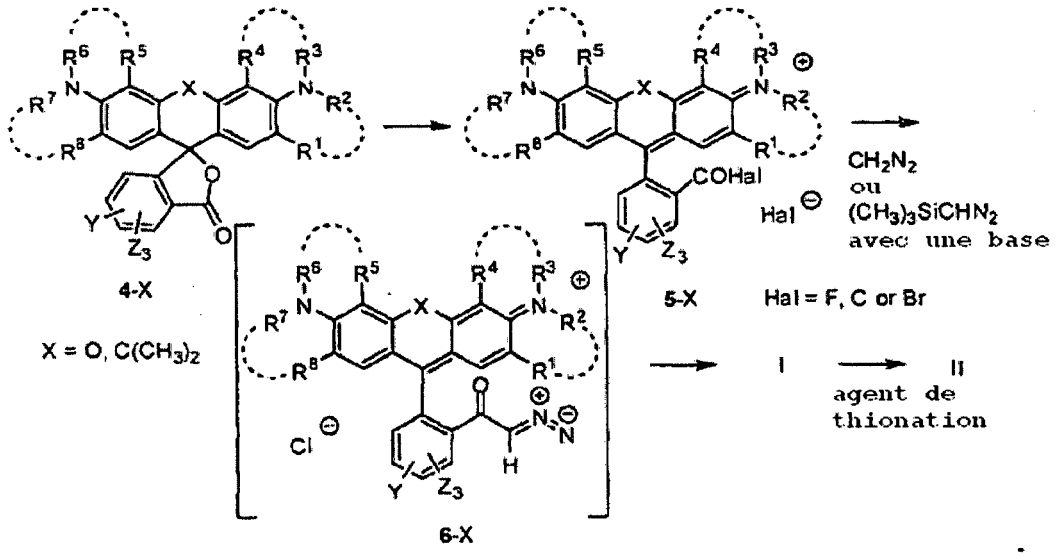
dans lesquelles R^1, R^2, (R^1-R^2), R^3, R^4, (R^3-R^4), R^5, R^6, (R^5-R^6), R^7, R^8, (R^7-R^8) = H, un groupe alkyle (cycloalkyle) non substitué ou substitué, cycloalcényle non substitué ou substitué, un résidu hétérocyclique non substitué ou substitué, aryle non substitué ou substitué, hétéroaryle non substitué ou substitué ; et X = O, S, $C(CH_3)_2$; Y = $COOR^9$, COCH=N=N, CH_2COOR^9, $N(R^1...R^2)$, $NR^{10}R^{11}$, SR^{12}, F, Cl ; Z = H, F, Cl ; W = O, S ; R^9 = H, un groupe alkyle non substitué ou substitué, cycloalkyle, ou un résidu hétérocyclique, *N*-succinimidyle, *N*-sulfosuccinimidyle, phényle non substitué ou substitué, aryle ou hétéroaryle ; R^{10}, R^{11} = C=S, C=O ; R^{12} = H, $(CH_2)_mCOOR^9$ (*m* = 1 à 11), alkyle non substitué ou substitué, cycloalkyle, un résidu hétérocyclique, aryle ou hétéroaryle.

3. Composé selon la revendication 2, choisi dans le groupe des composés 10, 17, 18, 22-Me, 23-Me, 22-Et, 23-Et, 22-H, 23-H, 22-NHS, 23-NHS, 25, 29, 30 et 31 ci-dessous



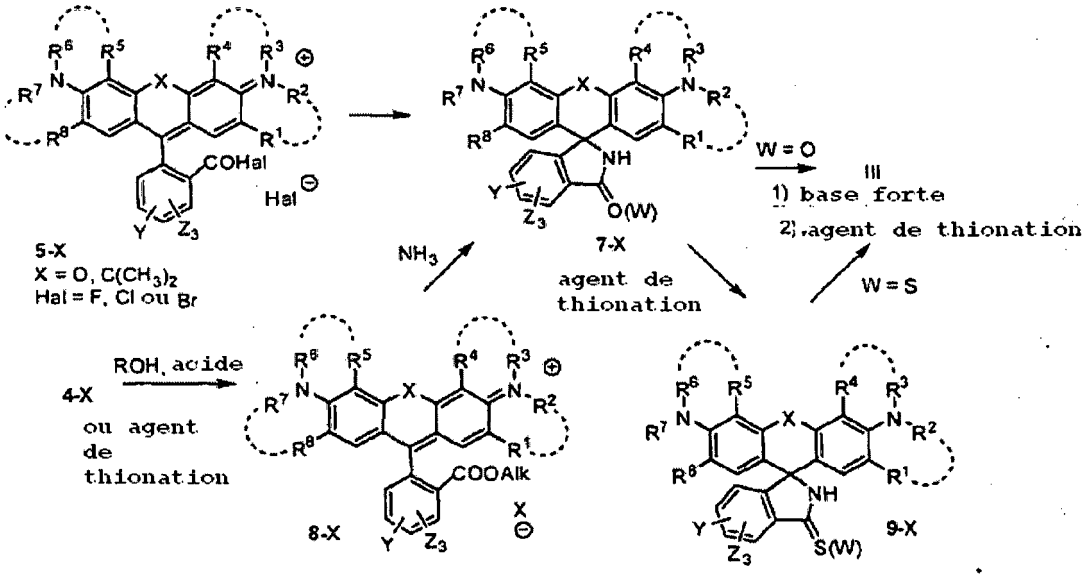
4. Procédé de préparation des composés I et II selon la revendication 2, comprenant les étapes de :

a) conversion du composé départ spiro-9*H*-xanthène ou 9,10-dihydroanthracène 4-X comme décrit dans le schéma suivant (ou leurs formes ouvertes équivalentes sur le plan de la synthèse présentant un groupe carboxylique libre) en halogénure d'acide 5-X correspondant ;
b) réaction du composé 5-X avec un composé de diazométhane pour produire la diazocétone I ;
c) éventuellement réaction du composé I avec un agent de thionation pour produire le dérivé de 1,2,3-thiadiazole II



5. Procédé de préparation des composés III selon la revendication 2, comprenant les étapes de :

a) conversion de l'halogénure d'acide 5-X ou de l'ester alkylique 8-X comme décrit dans le schéma suivant en l'amide 7-X ;
b) réaction du composé 7-X avec un agent de thionation pour produire le thioamide 9-X correspondant ;
c) déprotonation de l'amide 7-X ou du thioamide 9-X avec une base forte suivie de la réaction avec un agent de nitrosation



6. Utilisation des composés selon les revendications 1 à 3 comme réactifs pour une conjugaison ou une bioconjugaison.

7. Utilisation selon la revendication 6, dans laquelle la conjugaison ou la bioconjugaison comprend la formation d'au moins une liaison chimique covalente ou d'au moins un complexe moléculaire avec une entité ou une substance chimique, telle qu'une amine, un acide carboxylique, un aldéhyde, un alcool, un composé aromatique, un hétérocycle, un colorant, un acide aminé, un résidu d'acide aminé couplé à une quelconque entité chimique, un peptide, une protéine, un hydrate de carbone, un acide nucléique, une toxine, un lipide.

8. Utilisation selon la revendication 7, dans laquelle la conjugaison ou la bioconjugaison comprend la formation de deux liaisons chimiques covalentes et/ou complexes moléculaires ou plus.

9. Utilisation selon la revendication 8, dans laquelle les composés 22-NHS, 23-NHS, 17 et 30 avec deux groupes fonctionnels sont utilisés comme réactifs de réticulation (liaison).

10. Utilisation des composés selon l'une quelconque des revendications 1 à 3 ou de leurs conjugués pour une bioconjugaison avec des anticorps et pour un marquage immunologique.

11. Utilisation des composés selon l'une quelconque des revendications 1 à 3 ou de leurs conjugués comme colorants fluorescents photo-activables.

12. Utilisation des composés selon l'une quelconque des revendications 1 à 3 ou de leurs conjugués comme substances perméables à travers des cellules pénétrant à travers des membranes de cellules vivantes et fixées sous une forme non fluorescente masquée représentée par les formules structurelles G1 à G4.

13. Utilisation des composés selon l'une quelconque des revendications 1 à 3 ou de leurs conjugués tels quels ou après une photoactivation pour suivre et surveiller des processus dynamiques dans un échantillon ou dans un objet.

14. Utilisation selon la revendication 13, dans laquelle des changements de forme, de dimensions et/ou d'intensité du signal de fluorescence obtenus après une photoactivation des composés selon l'une quelconque des revendications 1 à 3 ou de leurs conjugués correspondent à des changements de l'échantillon ou de l'objet ou de son environnement.

15. Utilisation des composés selon l'une quelconque des revendications 1 à 3 ou de leurs conjugués tels quels ou après une photoactivation comme étiquettes dans des techniques de microscopie optique et d'imagerie, des dispositifs microfluides, une électrophorèse capillaire, des techniques de suivi de protéines.

16. Utilisation selon la revendication 15, dans laquelle les techniques de microscopie optique et d'imagerie comprennent la microscopie à déplétion par émission stimulée [STED], la « nanoscopie » à commutation de molécules simples (SMS) (résolution optique illimitée par diffraction par commutation de la fluorescence des molécules simples, telle que la microscopie de localisation de molécules simples [SMLM], la microscopie de localisation par photo-activation [PALM, PALMIRA, fPALM], la microscopie de reconstruction optique stochastique [STORM]), la spectroscopie par corrélation de fluorescence [FCS], la récupération de fluorescence après un photoblanchiment [FRAP], l'imagerie de temps de vie de fluorescence [FLIM], la dépletion de l'état de la terre avec un retour moléculaire individuel [GSDIM] et le transfert d'énergie par résonance de fluorescence [FRET].

17. Utilisation des composés selon l'une quelconque des revendications 1 à 3 ou de leurs conjugués tels quels ou après une photoactivation comme étiquettes spécifiques de mitochondries.

18. Utilisation selon la revendication 17, dans laquelle les composés 22-H, 22-NHS, 23-H et 23-NHS sont utilisés comme étiquettes spécifiques de mitochondries.

19. Utilisation des composés 22-H, 22-NHS, 23-H et 23-NHS dans une microscopie de localisation de molécules simples.

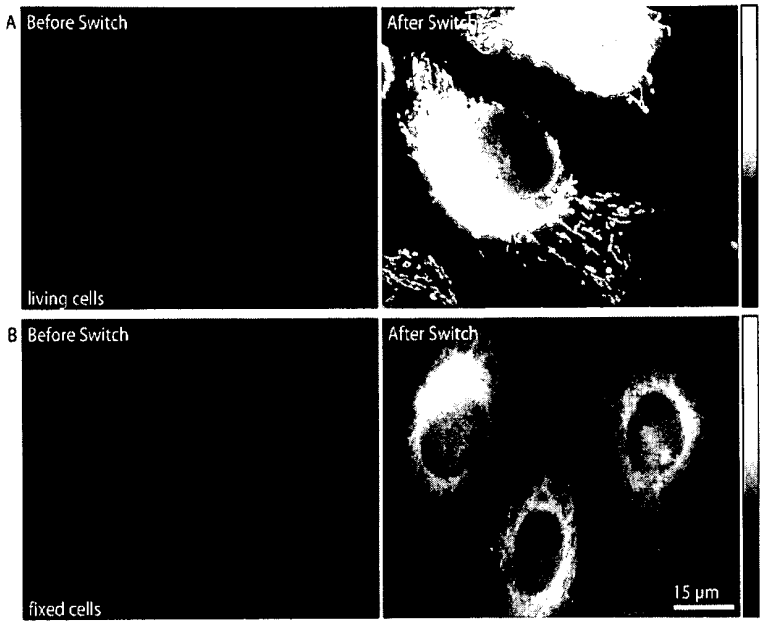

A
Before Switch
After Switch
living cells
B
Before Switch
After Switch
fixed cells
15 µm

Fig. 1

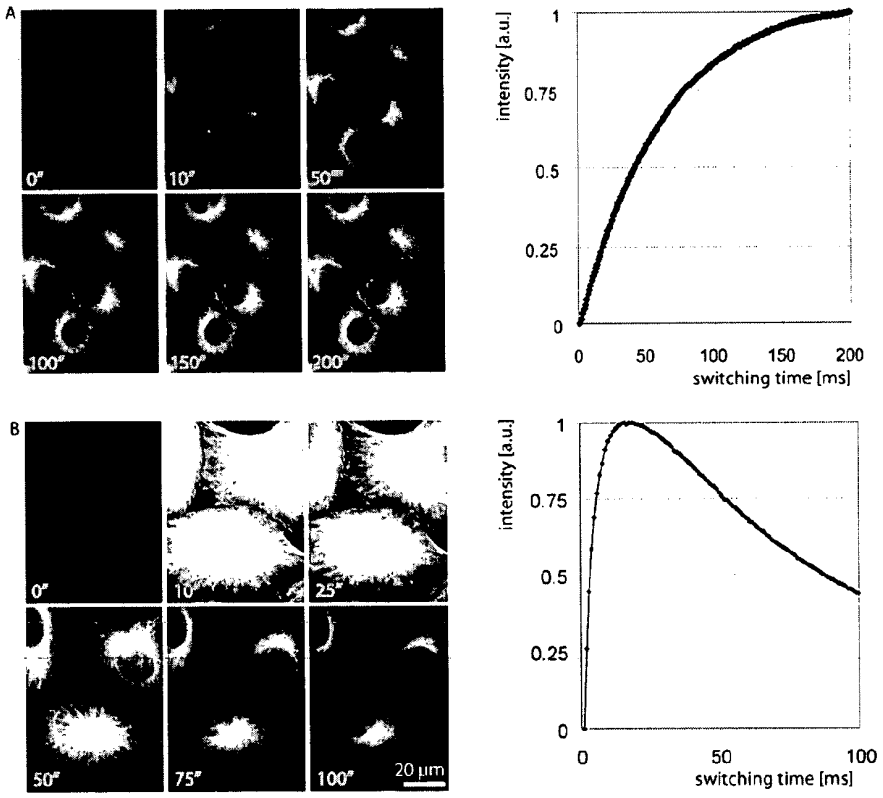

A
0"
10"
50"
100"
150"
200"
intensity [a.u.]
1
0.75
0.5
0.25
0
0
50
100
150
200
switching time [ms]
B
0"
10"
25"
50"
75"
100"
20 µm
intensity [a.u.]
1
0.75
0.5
0.25
0
0
50
100
switching time [ms]

Fig. 2

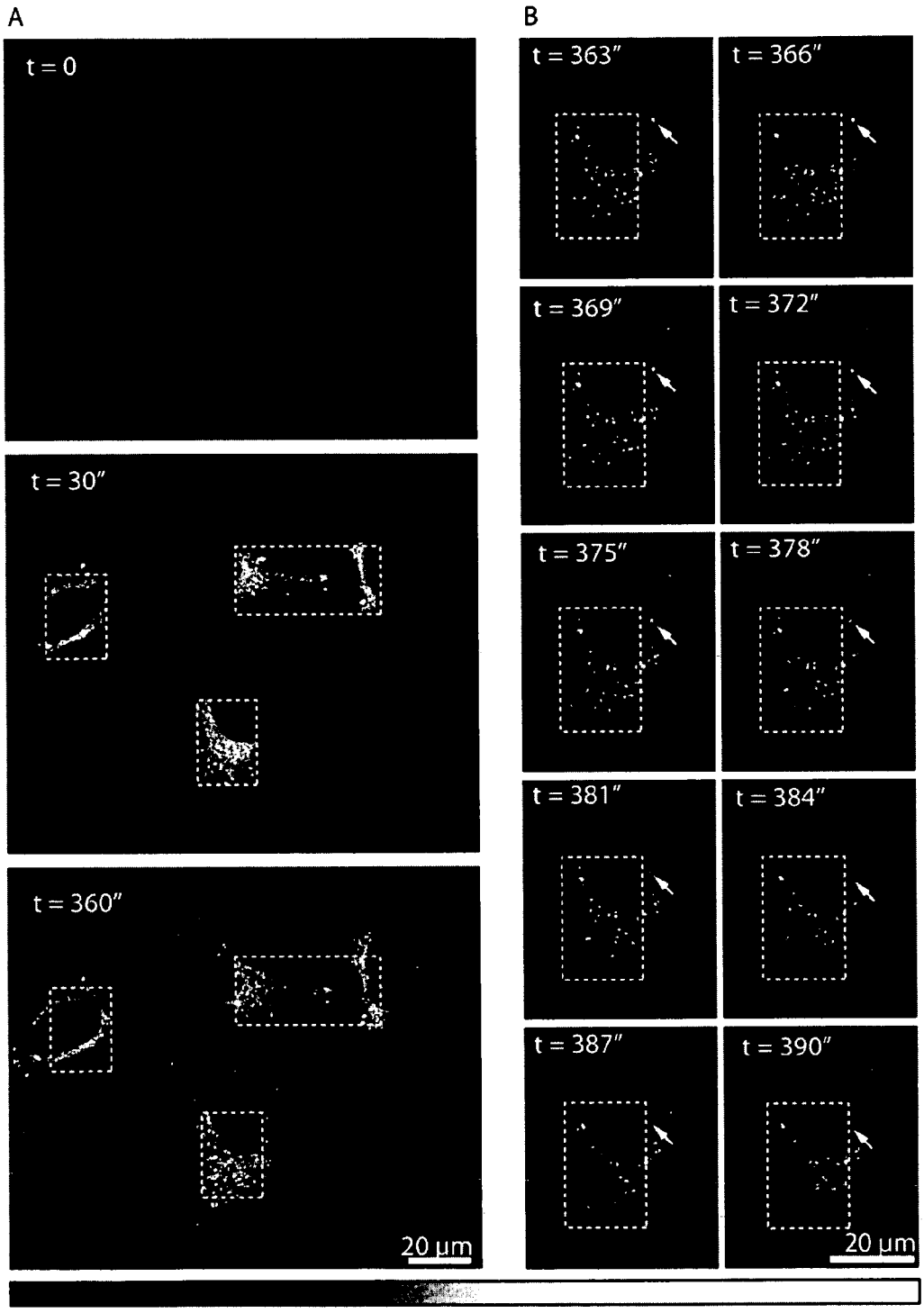

A
B
t = 0
t = 30″
t = 360″
20 µm
t = 363″
t = 366″
t = 369″
t = 372″
t = 375″
t = 378″
t = 381″
t = 384″
t = 387″
t = 390″
20 µm

Fig. 3

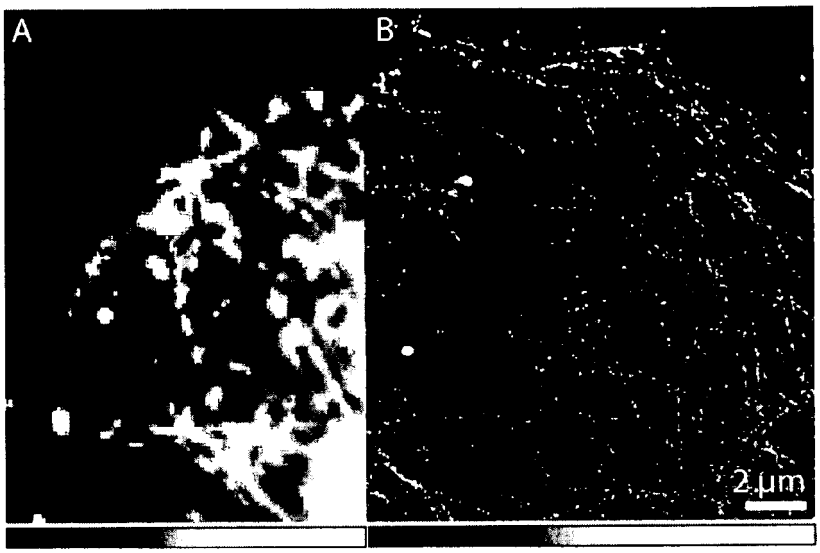


Fig. 4

REFERENCES CITED IN THE DESCRIPTION

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